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Ether Phospholipid Composition

of the Intestinal Mucosa

by

© M.A. Bates

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE AND NUTRITION

EDMONTON, ALBERTA

Spring, 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Effect of Diet on Ether Phospholipid Composition of the Intestinal Mucosa" submitted by M. A. Bates in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

To examine the effect of diet on ether phospholipid metabolism four groups of male Sprague-Dawley rats were fed one of four experimental diets for 28 days. Each diet contained 20% (w/w) fat from a mixture of tallow and safflower oil (T&SFO), safflower oil (SFO), a mixture of tallow, safflower and eicosapentaenoic acids (T&SFO&EPA) and a mixture of tallow, safflower and docosahexaenoic acids (T&SFO&DHA). Rats were fasted, sacrificed and the mucosa from the proximal and distal small intestine was collected as was the entire colon.

EPA increased alkenyl PE levels while DHA decreased them. Fishoil and SFO decreased alkenyl PC levels.

Feeding fish oil caused a significant reduction in C20:4(6) in both PC subclasses and a significant increase in C22:5(3) in diacyl and alkenyl PC. In the diacyl subclass of PE, EPA significantly reduced C20:4(6).

It was concluded that C20:4(6) availability is not the only factor regulating production of ether phospholipids.

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List of Abbreviations

pL phospholipid 1
GroPEtn alk-1'-enyl-glycero-3-phosphoethanolamine 2
E/CPG ethanolamine/choline phosphoglyceride 5
PAF platelet activating factor 5
DHAP dihydroxyacetone phosphate 9
PUFA polyunsaturated fatty acids 9
NADH reduced form of nicotinamide adenine dinucleotide 16
NADPH reduced form of nicotinamide adenine dinucleotide phosphate . . 16
ATP adenine triphosphate 16
CDP cytidine diphosphate 16
PC phosphatidylcholine 16
PE phosphatidylehanolamine 17
PLC phospholipase C 17
R generic fatty acid 18
G3P glycerol-3-phosphate 18
CoA coenzyme A 18
P/S polyunsaturated fatty acids / saturated fatty acid 23
T tallow 25
SFO safflower oil 25
DHA docosahexaenoic acid 25
EPA eicosapentaenoic acid 25
BHT butylated hydroxytoluene 26
BF_3 boron triflouride 26
EDTA ethylene diamine tetraacetic acid 27
HCl hydrochloric acid 27
KOH potassium hydroxide 27
KCl potassium chloride 27
ANSA 0.2% 1-amino-2-naphthol-4-sulfonic acid, 12% sodium metabisulfite and 1.2% sodium sulfite (anhydrous) 28
TLC thin layer chromatography 29
ANSA 8–anilino–1–naphthalene sulfonic acid in milliQ water 29
HPLC high performance liquid chromatography 30
B. cereus Bacillus cereus 30
NaOH sodium hydroxide 30
PEG polyethylene glycol 31
ANOVA analysis of variance 33
SNK Student-Newman-Kuels multiple range test 33

MUFA monounsaturated fatty acids 53

Chapter 1

Overview

1.1 Introduction

1.1.1 Definition of *ether phospholipid*

All biological membranes, eukaryotic and prokaryotic, contain phospholipids as their major structural lipids [104]. Phospholipids contain a phosphate group and the most important structural group in this class are the phosphoglycerides. Phosphoglycerides have a glycerol-3-P backbone with a fatty acyl chain attached at the sn-1 and sn-2 positions. The fatty acyl chain at position sn-1 is usually saturated while the fatty acyl chain at position sn-2 is usually unsaturated [104].

Ether phospholipids are much like glycerophospholipids except that there is an ether group attached at the sn-1 position as opposed to an ester-linked fatty acyl chain. The ether group in biological membranes is primarily an alkyl ether group or an alkenyl ether group. The double bond of the alkenyl moiety adjacent to the ether group has a cis configuration and the alkyl moiety adjacent to the ether group is usually saturated [76]. The ether lipids with O-alkyl groups are often called glycerol ethers and those with the O-alk-1'-enyl groups are called plasmalogens or vinyl ethers [76] (see Figure 1.1).

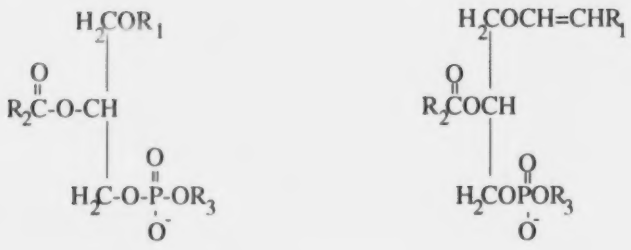

Figure 1.1: Structure of a glycerol ether and a vinyl ether.

1.1.2 Diet and ether phospholipids.

Fat can be a major part of the human diet comprising up to 50% of our calories. Approximately 95% of dietary fat is made of triacylglycerols and the nature and position of the fatty acid esterified to the glycerol backbone determines the characteristics of these dietary lipids [40]. By changing the type of fat in the diet one can manipulate the size and composition of the fatty acid pool in the body. Arachidonic acid (C20:4 (n-6)) has been found in high concentrations at the sn-2 position in ether phospholipids from all tissues and it has been shown that arachidonic acid levels of intestinal mucosa can be altered by diet [3]. With addition of eicosapentaenoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 (n-3)) to the macrophage-like P388Di cells Blank et.al. found that 50% of the alk-1'-enyl-glycero-3-phosphoethanolamine (GroPEtn) contained the hexaenoic species at the sn-2 position and the arachidonate content was greatly reduced [3]. Holub et.al. found that the alk-1'-enylacyl GroPEtn subclass of phospholipids was the largest reservoir of C20:5(n-3) and C22:6(n-3) in platelets from humans who had received dietary supplements of fish oil [43]. Since

arachidonic acid is common as a component of ether phospholipids it may be that arachidonic acid availability is the limiting factor in their production.

Although ether phospholipids are ubiquitous in the animal kingdom and minor amounts have been found in higher plants they have been largely ignored by nutritionists. Certain meats and seafoods can contain significant amounts of ether lipids and so need to be considered, along with dietary fat, as an important source of ether linked phospholipids [5].

1.2 Literature Review

1.2.1 Nomenclature

Naming of ether phospholipids is quite complicated mostly because of the difficulty found in distinguishing stereoisomers [48]. Rules that work for monoglycerols are not necessarily adequate for triacylglycerols. Also the stereospecificity of a glycerol molecule can and does change after certain reactions (ie phosphorylation) and so relationships between two molecules can be obscure. Different systems for the designation of stereoisomers have been chosen by various authors thus compounding the problem. The rules finally decided upon by the IUB-IUPAC are as follows:

1. Draw the glycerol molecule in a Fischer projection.

2. Place the secondary hydroxyl on the left of the middle carbon (the second carbon).

3. The carbon atom above the one attached to the secondary hydroxyl is carbon one and the carbon atom below is carbon three.

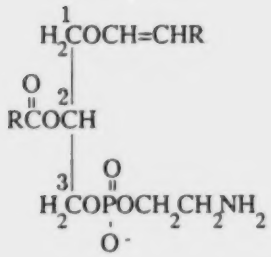

Figure 1.2: Chemical structure of the ethanolamine plasmalogen.

The use of this stereospecific numbering is indicated by *sn* before the stem name of the compound [48]. As long as the four bonds to carbon two remain intact the primary carbinol group will always have the same number no matter what the O substituent may be (see Figure 1.2).

When naming esters, ethers, or other O-derivatives the rules for naming carbohydrates are followed in addition to the rules mentioned above. Finally the prefix *rac-* is used before the full name if the product is an equal mixture of diametrically opposite isomers and the prefix X is used if the configuration of the product is unknown or unspecified [48].

There are two main ether linkages associated with glycerphospholipids in nature. They are the O-alkyl and the O-alk-1'-enyl moieties. The alkyl and alk-1'-enyl simply describe the type of bond between the one and two carbons of the aliphatic side chain adjacent to the ether linkage but indicate nothing about the rest of the molecule or about the saturation or unsaturation further along the aliphatic side chain. The double bond in the alk-1'-enyl linkage has a cis configuration [76]. Synonyms for the alkyl side chain are: glycerol ethers, saturated, and alkoxy. Synonyms for the O-alk-1'-enyl side chain are: plasmalogen, alkenyl, vinyl, enol, and unsaturated [56]. The O is used only if it is not clear that the aliphatic moiety is on glycerol.

Due to the confusion and cumbersome lengths of the chemical terms used for O-alkyl and O-alk-1'-enyl glycerolipids the IUB-IUPAC recommends use of the terms plasmanyl and plasmenyl for the 1-alkyl-2-acyl-*sn*-glycero-3-phospho- and the 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phospho- radicals respectively. Plasmanyl choline is equivalent to 1-alkyl-2-acyl-*sn*-glycerol-3-phosphocholine and plasmenyl ethanolamine is equivalent to 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine. The term phosphatidyl is only supposed to be used to designate the diacylglycerophospho - radical but commonly it is used for the mixture of diacyl, alk-1'-enylacyl, and alkylacyl compounds. A better term for this mixture is ethanolamine/choline phosphoglyceride (E/CPG) [46].

The major ether phospholipid of interest is the potent biologically active plasmalogen 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. Its common name is Platelet Activating Factor (PAF) [46].

1.2.2 Physical properties of ether phospholipids

The accepted model for the overall organisation of biological membranes is the fluid mosaic model. This model states that membranes are two dimensional solutions with lipids and globular proteins 'dissolved' throughout [78]. The bilayer is about 50Å thick and the essential repeating structural unit is the phospholipid [104]. Proteins either pass from one membrane of the bilayer to the other or are localised at one or the other of the two layers or are embedded in the hydrophobic matrix [104]. Some of the membrane lipids interact with membrane proteins and are necessary for protein function. According to this model the whole structure is dynamic; that is to say that the components are capable of lateral motion but they cannot rotate from

one membrane to the other. Fluidity of the membrane is influenced by fatty acid composition and cholesterol content.

Due to differences in the chemical and physical characteristics between ethers and esters it seems reasonable to assume that substitution of an ether linkage for an ester linkage in the phospholipid would effect the physicochemical nature of the membrane. However, knowledge of these effects is sketchy. Although ether linkages mainly affect the hydrophobic-hydrophilic interactions there are a number of ways that the ether linkage could affect the configuration of membranes. Ether lipids lack the carbonyl group at carbon one and therefore are less polar than their ester counterparts. Biological membranes containing significant amounts of ether phospholipids may have a lower dipolar membrane potential than membranes with ester phospholipids. A high quantity of ether phospholipids in biological membranes may also lead to reduced hydrogen bonding between the phospholipid and the hydrogen donors such as cholesterol, proteins, and water [56]. The lack of this carbonyl group also allows for closer packing in the plane of the bilayer of ether lipids which may influence the polar head group region [56].

The alk-1'-enyl phospholipid (the plasmalogen) has a unique double bond between the C1 and C2 of the aliphatic side chain adjacent to the ether linkage which can effect stereochemical relationships thus affecting the configuration of membranes [76]. With the exception of dialkyl-CPG, ether phospholipids have been shown in model membrane systems to lower the phase temperature of membrane bilayers. The dialkyl-CPG packs more closely in membranes suggesting less fluidity. The changes in packing effect the microenvironment of proteins and may alter membrane function. They have also been shown to decrease ion permeability of the membranes

when compared to their diacyl counterparts. The exception to this is the choline plasmalogen which seems to allow the chloride ion across readily [76].

The alkyl ether bond is resistant to enzymatic hydrolysis and the alk-1'-enyl ether bond is resistant to alkali and phospholipase action. Ether lipid metabolism is independent of ester lipid metabolism because ether linkages are formed and degraded by specific enzymes. This resistance to hydrolysis could also affect the function of the membrane. There has been some evidence that this selectivity by phospholipases in part depends on the source of the enzyme as well as the linkage at the sn-1 position [13].

It can be seen by the differences in chemical and physical properties between ether phospholipids and ester phospholipids that the substitution of ether phospholipids for ester phospholipids should affect the conformation and function of biological membranes. The exact mechanisms for these changes have yet to be elucidated.

1.2.3 Occurrence in Nature.

Ether phospholipids are abundant throughout the animal kingdom and have even been found in some higher plants. These lipids are found primarily in the membrane phospholipids. In mammals the greatest amount of ether phospholipid is in the nervous tissue, heart muscle, kidney, erythrocytes, testes, preputial glands, tumor cells, bone marrow, spleen, skeletal tissue, neutrophils, macrophages, platelets, lipoproteins [76], and leukocytes [82]. The liver generally has a low content of plasmalogens [36] while the lipoproteins from rat and human plasma contain quite high levels of plasmalogens (36% and 50% respectively) [76]. Approximately one fifth of the phospholipid in the human body are plasmalogens and about two thirds of

the total content of plasmalogens are found in the muscle [46]. Proportions of plasmalogen from skeletal muscle and heart varies significantly from species to species. Ethanolamine plasmalogens account for about 32-43% of the total phospholipid in the central nervous system and 27-30% in peripheral nerve myelin in the human. This value also changes from species to species depending upon degree of myelination and amount of white matter [46].

Ether phospholipids are also found in birds. Egg yolks from chicken eggs "contain small proportions of alkyl groups in the ethanolamine and choline glycerophospholipids and traces of ethanolamine plasmalogens" [46]. The salt glands of the herring gull and eider duck also have small amounts of choline and ethanolamine plasmalogens [46]. Other tissues from avian, marine, molluscan, protozoan, and bacterial species may contain a high proportion of ether-linked phospholipid. The halophilic, the methane producing, and the thermoacidophilic bacteria contain an unusual glycerolipid – a diphytanyl ether analogue of phosphatidylglycerophosphate [46]. This ether lipid has its ether linkages at the sn-2 and sn-3 positions. How these bonds are synthesised in the bacteria remains an enigma.

Ethanolamine and choline plasmalogens are also found in some plant tissue – peas, beans and seedlings. The alkenyl groups are mostly C16:0, 18:1, and 18:2 [46]. The plasmalogen content of pea and bean phospholipid is 0.1-0.2% [52].

Finally, ether phospholipids have been found in tumor cells. The major ether phospholipids found are the 1-alkyl-2,3-diacyl-*sn*-glycerols which exist in some tumor cells in a quantity that is substantially higher than that found in healthy cells. However, not all tumor cells have this lipid class in elevated amounts and staphylococcus infection has also been reported to cause elevated levels of this lipid so the

correlation between higher levels of 1-alkyl-2,3-diacyl glycerol and tumor cells is not exact [46]. In cells that have higher levels of these lipids it has also been found that the dihydroxyacetone phosphate pathway is very prominent [75]. Tumor cells seem to be low in the ethanolamine plasmalogen [96].

1.2.4 Function of ether phospholipids.

Ether phospholipids in mammalian cells tend to be concentrated in the plasma membrane and the ethanolamine plasmalogens tend to be situated on the inner surface of the membrane [46]. Apart from their roles as structural components and cell mediators the function of ether phospholipids remains an enigma. It has been suggested that some of the ether phospholipids associated with membranes act as storage sites for polyunsaturated fatty acids. Their ability to resist hydrolysis by phospholipase A2 seems to confer upon them a protective role [76]. Some plasmalogens exist in clusters associated with proteins and may have a role in cation and anion leak [46].

Arachidonic acid and precursors for eicosanoid production.

The term eicosanoid signifies a group of 20 carbon fatty acids that include leukotrienes, prostaglandins, and thromboxanes. They are regulators of vascular smooth muscle contractility, hypertrophy, and cell proliferation [26]. Arachidonic acid is the major precursor for eicosanoids and it is released, upon stimulation, from phospholipids by the action of phospholipase A2. Various cell types can be stimulated by either bradykinin, angiotensin II, antidiuretic hormone or a protease like thrombin [76]. The enzymes involved in converting arachidonic acid to eicosanoids are cyclooxygenase, lipoxygenase, and cytochrome P450 epoxygenases. These pathways are known

collectively as the "arachidonate cascade" [76].

Ethanolamine plasmalogens appear to act as storage sites for arachidonic acid, especially the subclass 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine [2]. In inflammatory cells the 1-alkyl- and the 1-alk-1'-enyl- linked ether phospholipids are quite pronounced and are often the major pools for arachidonic acid in these cells [15]. For example in the human neutrophil 71% and 66% of the total arachidonate in ethanolamine and choline linked phospholipids is found in the 1-alk-1'-enyl-2-arachidonoyl-ethanolamine phosphoglyceride and 1-alkyl-2-arachidonoyl-choline phosphoglyceride respectively [15]. It is the 1-O-alk-1'-enyl group that contains more than 50% of the cellular arachidonic acid [86]. Tessner et.al. showed that the deacylation of phosphatidyl ethanolamine in ionophore-stimulated neutrophils was selective for arachidonic acid-containing species of ether phospholipids and that resting neutrophils acylated [3H]-alkenyllyso-ethanolamine phosphoglycerides by using predominantly arachidonic acid. This was also true for alkyllyso-choline phosphoglycerides [86]. Chilton demonstrated that the human neutrophil can use large stores of arachidonic acid in 1-ether linked phospholipids for leukotriene biosynthesis. He could not, however, tell if this arachidonic acid came from only one 1-ether linked subclass or from a mixture of 1-acyl- and 1-ether linked subclasses [15].

Gross and Ford showed that in intact vascular smooth muscle the majority of arachidonic acid is present in plasmenylethanolamine and that this arachidonic acid is rapidly mobilised after stimulation by angiotensin II [26]. In the rabbit aorta $> 63\%$ of the plasmalogens contain arachidonic acid esterified to the sn-2 position. It seems that there is a specific membrane bound calcium independent phospholipase A2 that, upon stimulation, uses arachidonic acid containing plasmalogens preferentially

as substrates [26].

Human platelets contain significant amounts of 1-O-alkyl-2-acyl-choline phosphoglycerides (10% of the total phosphatidyl choline fraction) and 1-O-alk-1'-enyl-2-acyl-ethanolamine phosphoglycerides (60% of the phosphatidyl ethanolamine fraction) [60]. Arachidonic acid comprises 25% and 64% of all the fatty acids associated with phosphatidyl choline and phosophatidyl ethanolamine respectively [60]. Of all the arachidonic acid link to phosphatidyl choline 17% was found in the 1-O-alkyl-2-acyl choline phosphoglyceride pool [60]. In contrast, of all the arachidonic acid linked to phophatidyl ethanolamine 65% was in the plasmalogen fraction [60]. The 1-O-alkyl-2-acyl-choline phosphoglycerides are used to synthesise PAF by a deacylation-reacylation pathway. The action of phospholipase A2 on 1-O-alkyl-2-acyl-choline phosphoglyceride would yield precursors for PAF production and products of the arachidonic acid cascade (eicosanoids) [60].

It is known that ether phospholipids contain substantial amounts of arachidonic acid in a variety of tissues. However, whether or not the arachidonic acid from these ether phospholipids is the only arachidonic acid used for eicosanoid biosynthesis is not clear. All the phospholipid classes can contain arachidonic acid at the sn-2 position and so the specific molecular species from which arachidonic acid is hydrolysed for eicosanoid production is not clear. More work needs to be done in this area, however, under first consideration it would seem that the ether phospholipids, especially the plasmalogens, are important contributors to the arachidonic acid pool.

PAF synthesis.

PAF is a special class of ether phospholipids. It has been identified as 1-O-alkyl-2-acetyl-choline phosphoglyceride [60]. In neutrophils it causes aggregation and degranulation, it regulates chemotaxis and chemokinesis, increases cell adherence, enhances respiratory bursts and peroxide production, and stimulates the production of arachidonic acid metabolites formed via the enzyme lipoxygenase. In platelets it causes aggregation and degranulation, stimulates uptake of calcium, stimulates protein phosphorylation, the phosphatidyl inositol cycle and arachidonic metabolism thus potentiating the formation of hydroxy fatty acids and thromboxanes. PAF increases vascular permeability, constricts ileum and lung strips, stimulates hepatic glycogenolysis, and causes bronchioconstriction, increases pulmonary resistance, pulmonary hypertension and edema, reduces lung compliance, causes neutropenia and thrombocytopenia, causes intestinal necroses, and produces systemic hypotension [76]. It has been suggested that PAF is a mediator of inflammation and anaphylaxis [79].

Arachidonic acid release and metabolism is tightly coupled to PAF synthesis. PAF is synthesised by two pathways; de novo and remodeling. The de novo pathway may be responsible for maintaining endogenous physiological levels of PAF necessary for certain cellular functions but the remodeling pathway is the source of PAF in hypersensitivity responses [91]. In the remodeling pathway a variety of cells are activated and as a result 1-O-alkyl-2-acylglycerophoshocholine is deacylated at the sn-2 position by phospholipase A2/transacylase yielding lysoPAF which is then acetylated to produce PAF. Phospholipase A2/transacylase is highly specific for the 1-O-alkyl-2-acylglycerophosphocholines [76]. This enzyme complex may reside in a

single protein or as a tightly integrated complex called CoA-independent transacylase [91]. In this pathway bioactive arachidonic acid for eicosanoid synthesis can also be produced [76]. It seems that lysoplasmalogens are branch point intermediates as they are needed for PAF production but may also reincorporate arachidonic acid for redistribution to phospholipids thus affecting eicosanoid biosynthesis.

Another precursor to PAF is 1-alkenyl(alkyl)-ethanolamine phosphoglyceride. According to Sugiura et.al. 1-alkenyl(alkyl)-ethanolamine phosphoglyceride causes degradation of cellular alkylarachidonoyl-choline phosphoglycerides by a cofactor-independent transacylation system which may increase lyso PAF availability for PAF production [80]. The exact function of the ethanolamine plasmalogen is unclear, however it is suggested that it acts as a storage site for precursors for PAF synthesis [80].

Scavengers for oxygen generated free radicals.

Phagocytosing leukocytes convert O_2 to O_2^-, H_2O_2, OH· and $^1O_2$. These biologically active species can cause tissue damage during inflammation. The cell death in tissue damage is due to the action of these radicals on lipids, proteins and nucleic acids [58]. Plasmalogens are extremely vulnerable to oxidation by singlet oxygen and/or radicals. This was shown by Morand et.al. in an experiment that indicated that the plasmalogens were rapidly and selectively destroyed by irradiation with long wavelength ultraviolet light [58]. They demonstrated that mutant cells that lacked plasmalogens were much more sensitive to death by irradiation than were wild type cells. This suggested to Morand et.al. that plasmalogens may act as scavengers of reactive oxygen species to protect other targets within the histidyl, methionyl and

tryptophanyl residues of membrane-spanning proteins [58].

Zoeller et.al. found that if the activities of the two enzymes that catalyse the first two steps of plasmalogen biosynthesis are low in Chinese hamster ovary cells then the plasmalogen content of these cells is also low and these cells are more susceptible to irradiation damage than are wild type cells [102]. These two enzymes are dihydroxyacetone phosphate acyltransferase and alkyl synthase and are located in the peroxisomes of normal cells. Since all other enzymes involved in plasmalogen biosynthesis are located on the endoplasmic reticulum it is possible to restore normal plasmalogen levels of these mutant cells by supplementation of the growth medium with 1-O-hexadecyl-*sn*-glycerol and this supplementation restores considerable resistance to irradiation [102]. This supports the conclusion made by Morand that plasmalogens serve as scavengers of reactive oxygen species and thus protect membrane proteins from irradiation [58]. The ability to restore normal cell resistance to singlet and/or radical oxygen damage by supplementation with 1-O-hexadecyl-*sn*-glycerol has significant implications for the treatment of Zellweger syndrome [42].

It is the oxidation of low density lipoproteins (LDL) that allows their uptake by macrophage which, once in the foam cell stage, are atherosclerotic. The presence of the ethanolamine plasmalogen in nascent LDL may serve as an antioxidant protecting the lipoprotein from oxidation and eventual uptake by the macrophage [92]. It is the lack of the plasmalogen that may cause the LDL to be atherogenic and so the role of plasmalogens as oxygen scavengers has major implications in the treatment of heart disease.

Membrane fluidity.

The structure of plasmalogens was discovered over 30 years ago but since then their function as membrane constituents has remained a mystery. Most phospholipids have a carbonyl group in the sn-1 position but the plasmalogens are missing this group and instead have an ether linkage. Plasmalogens also have a double bond between the carbon one and carbon two of the aliphatic side chain adjacent to the ether linkage. This double bond may influence the fluidity of the cellular membrane. It has been found that the transition temperature of membranes containing ethanolamine plasmalogens is lower than that of membranes lacking in plasmalogens suggesting that the presence of plasmalogens reduce membrane fluidity [76]. It is not known the exact mechanism by which this happens.

Hermetter et.al. measured fluorescent anistropy in plasmalogen depleted cells and control cells from cultured fibroblasts. They found that the higher the plasmalogen content the lower the fluidity of the membrane [41]. This has important implications for the proper functioning of membranes since most membrane bound proteins require optimal fluidity for proper functioning [74]. The ability of proteins to migrate laterally within a membrane seems to be the mechanism needed for the regulation of specific cell surface properties [16]. Changes in lipid composition need only be small to affect protein function if the change affects the microenvironment of the protein [16]. If it is true that plasmalogens reduce membrane fluidity then there are implications for diseases such as leukemia where it has been shown that successful antileukemic treatment resulted in an increase in membrane microviscosity [103]. Studies on membrane composition and fluidity may also be important for the development of aging as it has been found that altered lipid-protein interactions

appear to play a role [103].

1.2.5 Biosynthesis of ether phospholipids PE and PC.

In order to look at the biosynthesis of ether phospholipids we need to look back at glycolysis. It is the glycolytic pathway that supplies the dihydroxyacetone phosphate (DHAP) necessary for the first step in the synthesis of the alkyl ether species of phosphatidic acid in eukaryotes. The DHAP is acylated in the endoplasmic reticuluum or the peroxisomes by the enyme DHAP acyltransferase to produce 1-acyl DHAP [76][104], see Figure 1.3. The microsomal enzyme uses either DHAP or glycerol-3-phosphate while the peroxisomal one only uses DHAP [76]. The acyl group is then exchanged for an alkyl group donated by an alcohol. The alcohol was formed by the reduction of an acyl-CoA with two moles of NADPH or NADH catalysed by the enzyme acyl-CoA reductase which prefers saturated substrates over unsaturated ones [76]. 1-alkyl-DHAP is then reduced with NADPH [76][104] leaving an hydroxyl group at carbon two. This alcohol is subsequently acylated by a long chain CoA to produce 1-alkylglycerophosphate (the alkyl ether derivative of phosphatidic acid). At this point dietary ether lipids can enter the pathway because ether phospholipids are catabolised during absorption to produce alkylglycerols which are phosphorylated by an ATP:alkylglycerol phosphotransferase to form 1-alkyl-2-lyso-*sn*-glycerol-3-P [76]. To make the ether linked phospholipid the 3-phosphate group is removed leaving 1-alkyl-2-acyl glycerol which then reacts with CDP-choline or CDP-ethanolamine to form phosphatidylcholine or phosphatidylethanolamine respectively. A microsomal enzyme, 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine Δ-1-desaturase, desaturates phosphatidylethanolamine to phosphatidalethanolamine (the ethanolamine

plasmalogen).

The Δ-1 desaturase enzyme does not use 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine as a substrate and so far the mechanism by which the choline plasmalogen is synthesised is a mystery. It has been suggested that the ethanolamine plasmalogen is a precursor to the choline plasmalogen by various reactions. These reactions involve CDP reverse substitution of P-choline for P-ethanolamine, base exchange, methylation of plasmalogens, or a coupled reaction sequence catalysed by phospholipase C and CDP-choline cholinephosphotransferase [76].

1.2.6 Transport of ether phospholipids.

After phospholipids are synthesised they are sorted and directed to their appropriate destinations either within the cell or to the extracellular environment [29]. Since most phospholipids are synthesised in the endoplasmic reticuluum there must be a mechanism to transfer the lipids to other parts of the cell [104]. How this transport occurs is not well defined but it has been suggested that the exchange of phospholipid species between two membranes occurs via phospholipid exchange proteins [104]. These were discovered in rat liver in 1968 by Wirtz and Zilversmit and since then many have been found in various tissues [76]. The phospholipid transfer proteins fall into three main categories: 1) proteins specific for phosphatidylcholine, 2) proteins that are highly active with phosphatidylinositol and 3) proteins that are active with most phospholipids and cholesterol (nonspecific transfer proteins) [85]. In the first two categories the proteins form 1:1 complexes with the specific phospholipid class and transfer the phospholipid between membranes [85]. The exact mechanism for this action remains largely unknown. Szolderits et.al. suggest that it has something to do

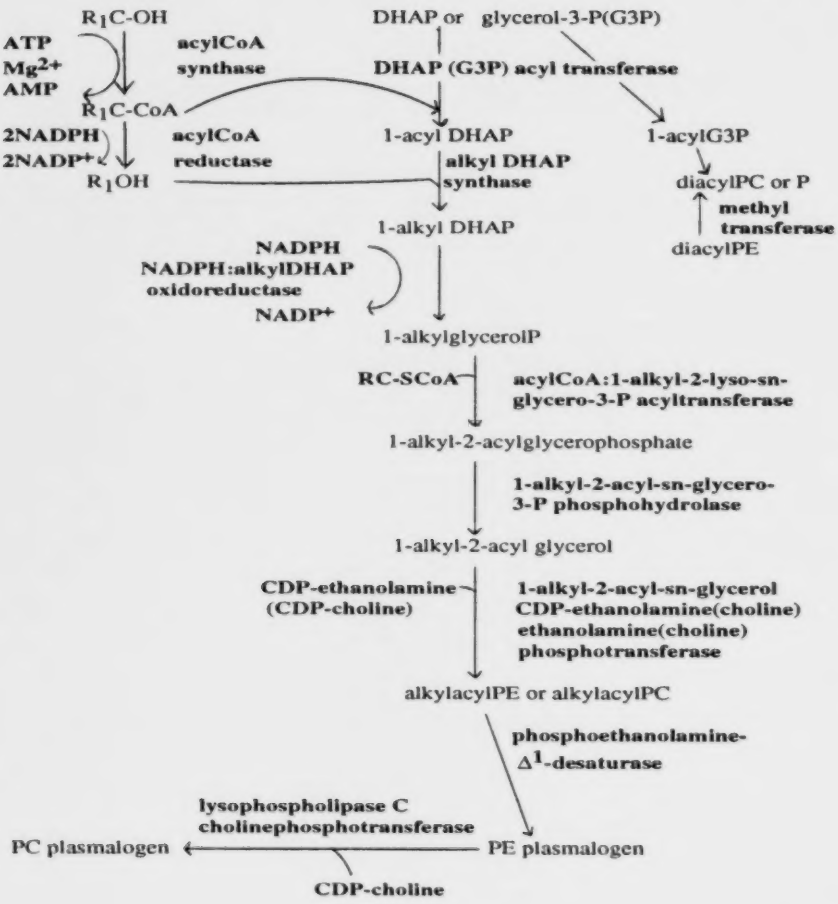

Figure 1.3: Biological pathway for the biosynthesis of ether phospholipids.

with polar head group recognition and penetration of the protein into the membrane [85]. It is into the hydrophobic/hydrophilic interface that these proteins penetrate during a transfer event and so any changes in phospholipid composition that affect the physicochemical properties of the membrane could influence phospholipid transport [85]. It has been found that ether phospholipids are transferred at a higher rate than their diacyl counterparts [85]. This may be due to the replacement of the ester bond by an ether bond which would reduce the polarity of the membrane causing a less defined hydrophobic/hydrophilic boundary [85].

Proteins in category three mediate transport probably by the formation of membrane contact. Their action appears to be independent of phospholipid head group recognition and binding but the mechanism is unknown [85].

For the membrane to grow a net transfer of lipid is required. There has been no evidence to indicate that these transfer proteins are responsible for this job [76]. The mechanism for the net transfer of lipids is still under investigation.

1.2.7 Ether phospholipids and disease.

Ether phospholipids are minor constituents of all human tissue and yet they have been implicated in the progression of various disease states. Although the biosynthesis and metabolism of ether phospholipids is fairly well understood only PAF has been shown to have a discrete biological role. It is suspected that other ether phospholipids, especially the plasmalogens, have a role but the exact nature of this role has remained elusive.

Zellweger disease is a genetic disorder in infants. A lack of peroxisomes in the liver and kidney of these children characterises this disease [42]. It is lethal and

infants with Zellweger's disease live for only a few months. Without peroxisomes the cell cannot make plasmalogens because the first two enzymes required are missing. Heymans et.al. have shown that the reduction in plasmalogen content is due to a defect in the alkyl ether phospholipid synthesis and not to a specific defect in plasmalogen synthesis [42]. It is possible that this reduction or absence of tissue plasmalogen may be sufficient to create the abnormalities seen in Zellwegers patients. This may prove valuable as it is thought that exogenous plasmalogens may correct the plasmalogen levels in these patients [42]. There may be other metabolic pathways present in peroxisomes which, in their absence, may be responsible for this disease but none are known. For this reason it seems that the continued study of plasmalogens and their biological function in this disease is warranted.

Plasmalogens and other ether phospholipids have also been found in significant amounts in cancer cells [69][99]. The 1-alkyl-2,3-diacyl-*sn*-glycerols have been proposed as tumor markers because of their high level in hepatocellular carcinoma cells [46]. The proportion of alkylacyl-choline phosphoglycerides is elevated to 0.8-3.5% in three to five tumor types. It is suggested that they are present as antitumor agents. Ether phospholipids are cytotoxic and induce tumor cell differentiation and modulate the host immune response [69]. Experiments done with patients suffering from cancer of the uterine cervix involved feeding these patients 1-alkylglycerols from the liver of a Greenland shark (0.6 g/day). This treatment affected the tumors and reduced mortality [55]. This affect may be caused by the dead cancer cells in inflamed cancer tissues being ingested by macrophage which then present antigens to the B-lymphocytes and specific antibodies are made [99]. In this case the cancer cells are immunogenic. This has major implications in cancer research and should

be studied further.

Plasmalogens make up 30-40% of the phospholipids present in the heart [102]. Their degradation products are lysophospholipids and it is known that ischemic myocardium accumulates lysophospholipids. This accumulation determines the electrophysiologic dysfunction that is manifest in ischemic myocardium [72]. Lysophosphoglycerides are produced either by the action of a cytosolic calcium independent phospholipase A2 on plasmalogens or by singlet oxygen or free radical oxidation of the vinyl-ether linkage in plasmalogens [72]. The singlet oxygen can be generated by human eosinophils [49] or by xanthine oxidase [67]. Since plasmalogens may play a role as scavengers for oxygen generated free radicals they deserve closer examination with regard to their role in myocardial ischemia. In the same way plasmalogens may play a role in the sequelae of intestinal ischemia [67] and therefore should be studied further. This free radical scavenging may also have implications in atherosclerosis via the mechanism by which it prevents the oxidation of low density lipoproteins thereby blocking uptake by the macrophage.

1.3 Summary and Rationale for study.

Ether phospholipids are ubiquitous in the animal kingdom. They are present to varying degrees in the membranes of cells in all tissues in the human body. These lipids have different chemical and physical properties than their ester counterparts and so it is reasonable to assume that they have different affects on the physicochemical nature of biological membranes. By changing the phospholipid composition of the membrane one may see alterations in protein function, ion channels and membrane

fluidity. All of these changes could be deleterious, causing the inception of diseases such as cancer, atherosclerosis, inflammatory diseases, and Zellwegers disease. It seems impossible to believe that cells would go through all the trouble to make ether phospholipids if they had no specific role for them however, with the exception of PAF, no specific role other than a structural role has been proven. Plasmalogens may act as storage sites for arachidonic acid which is a precursor to PAF and eicosanoids, as scavengers of free radical oxygen and they may influence membrane fluidity. These roles would have significant implications in the study of diseases and their cures and so warrant further research.

Phospholipids are available to the body through bile, diet and the sloughing off of epithelial cells [29]. Ether phospholipids constitute a significant proportion of the total phospholipids in animal and human tissues. A high proportion of the ethanolamine plasmalogens have arachidonic acid at their sn-2 position (45% in the intestinal mucosa)[101]. The eicosapentaenoic acid in fish oil may compete with arachidonic acid for incorporation into phospholipids and at the level of cyclo-oxygenase in the formation of eicosanoids [28]. It has also been shown that fish oil reduces the synthesis of arachidonic acid by inhibiting the Δ-6-desaturase enzyme. Both of these actions affect eicosanoid production [28]. This lower eicosanoid production should have a positive affect on inflammatory diseases and therefore merits further study.

1.4 Outline of Thesis.

1.4.1 Hypothesis.

Due to the effects of fish oil on the arachidonic acid production and subsequent eicosanoid synthesis and knowing that a high proportion of ether phospholipids (especially the plasmalogens) contain arachidonic acid at the sn-2 position of the glycerol backbone it is reasonable to assume that the biosynthesis of ether phospholipids is influenced by the arachidonic acid availability. Therefore the hypothesis of this study is that arachidonic acid is the limiting factor in ether phospholipid biosynthesis in intestinal mucosa.

1.4.2 Statement of Objective

The main objective of this study is to examine if diet induced changes in arachidonic acid content alter ether phospholipid levels in the intestinal mucosa. The model will be male Sprague-Dawley rats which will be fed diets with varying P/S ratios and fish oil content.

1.4.3 Thesis Organisation

Chapter I is an indepth review of the literature pertaining to the metabolism and synthesis of ether phospholipids. The design of the experiment is given in chapter II. The results and a discussion of the results as well as a general discussion pertaining to the overall study and suggestions for future research are given in chapter III.

Chapter 2

The Experiment

2.1 Introduction

Ether phospholipids are found in the membranes of virtually all animal cells [6]. With the exception of PAF, a definite functional role for ether phospholipids has yet to be determined. There is evidence to suggest that they play a role in the maintenance of physico–chemical properties of membranes, they may act as storage sites for arachidonic acid (the precursor for eicosanoid production) [45], and they may be antioxidants [67]. Ether phospholipids have been implicated in the progression of various disease states [42],[69], [67], [72], and therefore warrant further study. The main fatty acid found at the sn–2 position of ether phospholipids in intestinal mucosa is arachidonic acid (45%) [101]. It is known that dietary fat can alter membrane phospholipid composition in the intestinal mucosa [22], [2]. Thus by limiting arachidonic acid availability through diet fat composition it is possible that ether phospholipid production will be affected. The purpose of this study is to determine whether or not ether phospholipid content in intestinal mucosa is affected by limiting arachidonic acid availability.

2.2 Materials and Methods

Male Sprague-Dawley weanling rats weighing between 48 and 62 grams were randomly divided into four groups with seven rats in each group such that the average body weight per group was similar. The average body weight of the rats at this time was 56.8±0.6 grams. Each group was fed rat chow for at least four days before being fed one of four nutritionally complete semi-synthetic diets containing 20% fat (w/w) for four weeks, (see Table 2.1) [32]. The fat was provided by tallow and safflower oil (T&SFO), safflower oil (SFO), tallow and safflower oil and docosahexaenoic acid (T&SFO& DHA), or tallow and safflower oil and eicosapentaenoic acid (T&SFO& EPA) [32]. The DHA and EPA were obtained from Japan and the safflower oil was from Safeway. Not all rats were started on their respective diets simultaneously but were staggerred so as to avoid having to sacrifice all the rats on the same day. The rats were housed individually and were given diet and water ad libitum. Food and feed cups were changed every second day. The diet is shown in Table 2.1 and the fat composition for each diet is illustrated in Table 2.2.

Table 2.1: Composition of each diet. Each group was fed one of the four diets shown. The tallow and safflower oil diet is indicated by T&SFO, the safflower oil diet is indicated by SFO, the tallow and safflower oil and fish oil diets are indicated by T&SFO&DHA or EPA.

Ingredients	*Diet Group*			
	T&SFO	SFO	T&SFO &DHA	T&SFO &EPA
basal diet[a]	800 g	800 g	800 g	800 g
tallow	180 g	–	130 g	130 g
safflower oil	20 g	200 g	20 g	20 g
fish oil (25% DHA)	–	–	50 g	–
fish oil (EPA)	–	–	–	50 g

[a]Basal diet consisted of 270g high protein casein, 2.5g L–Methionine, 208.5g dextrose, monohydrate, 200g cornstarch, 50g cellulose, 50.8g mineral mix–BT (170750), 0.3g sodium selenite (0.0445% Na_2SeO_3 in sucrose), 0.23g manganese sulfate ($MnSO_4.H_2O$), 10g vitamin mix, A.O.A.C. (40055), 6.25g inositol, 1.4g choline chloride. Each diet had a caloric content of 4.5Kcal/gram diet.

Diet was prepared weekly and stored at −20°C . The amount of food eaten by each rat was monitored during week one and week four. Animals were weighed at the beginning of their dietary treatment and body weight was monitored weekly.

2.2.1 Diet Analysis

Lipids were extracted from each diet according to the Folch extraction method [25]. Briefly, one half gram of the diet was mixed with twenty mls 2:1 chloroform–methanol mixture (v/v) containing 50 mg/L of the antioxidant butylated hydroxytoluene (BHT). The crude extract was mixed with 0.2 its volume of 0.88% saline (w/v). One ml of the bottom layer was dried under nitrogen. Fatty acid methyl esters were then prepared using the method of Morrison [59]. The reagent was boron trifluoride–methanol (BF_3–methanol) and the mixture was heated to 100°C. The

tube was cooled and the esters were extracted with water and hexane. The top layer was dried under nitrogen and reconstituted in one ml hexane for gas liquid chromatography using a Perkin–Elmer gas chromatograph, model 8320. A PEG fused silica polar capillary column, Stabilwax, was used with a column length of 30 m and an internal diameter of 0.3 mm. The column was manufactured by Bellfonte, TA. The carrier gas was helium. A split ratio of 100:1 was used and the flow rate was 1 ml/min. Fatty acid composition of each experimental diet can be found in Table 2.2.

2.2.2 Tissue Collection

At the end of four weeks the rats were fasted overnight and sacrificed using ether. It was discovered that one rat in each of the T&SFO diet group and the T& SFO&EPA diet group was a female and so was discarded. The proximal and distal part of the small intestine , minus the middle 20cm, and colon was collected and placed into cold saline. The proximal small intestine included the duodenum. Each tissue was washed three times with cold saline (0.88% w/v), placed onto an ice cold glass plate and kept moist with saline. The fat was carefully stripped away from the tissue. The proximal and distal part of the small intestine were cut laterally, the mucosa was gently blotted dry with kimwipes, collected by scraping and placed into a preweighed and labelled scintillation vial containing approximately five mls of sucrose–EDTA buffer (0.25 M sucrose, 0.10 M Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.2 with KOH/KCl [7]. The vials were again weighed to determine the wet weight of the mucosa. For the colon, the fat was stripped away and the tissue was cut open and placed into a preweighed and labelled scintillation vial containing about 5 mls

of sucrose–EDTA buffer. All vials were stored at −70°C until needed.

2.2.3 Lipid Extraction

Lipids were extracted from the tissue according to the method of Folch (see above) [25]. A Polytron homogeniser was used to homogenise the tissue. Centrifugation was used to separate the layers upon extraction. The bottom layer was collected, transferred into a 16 × 100 mm screw cap culture tube, evaporated to dryness under nitrogen at about 50°C and resuspended in one ml of hexane. These tubes were capped with teflon lined lids and stored under nitrogen at −70°C until needed for lipid analysis.

2.2.4 Lipid Phosphorus Analysis

Lipid phosphorus analysis was done both before and after thin layer chromatography by the method of Bartlett; modified by Marinetti (1962) [57]. Duplicate 50μl, 100μl, 150μl, 200μl and 250μl of PC standard (1 mg/ml) were placed into labelled tubes. Duplicate 25μl samples of the proximal small intestine and 50μl of the distal small intestine and colon were placed into labelled tubes. Each tube was dried under nitrogen and 0.9 ml Perchloric acid was added. Digestion was carried out for 20–30 minutes in a hot sand bath (150–200°C). Aluminum foil was used as a cover to minimise perchloric acid fumes. Upon cooling, 7.0 mls of milliQ water, 0.5 ml of 2.5% (w/v) Ammonium Molybdate and 0.2 ml ANSA (0.2% (w/v) 1–amino–2–naphthol–4–sulfonic acid, 12% (w/v) sodium metabisulfite and 1.2% (w/v) anhydrous sodium sulfite; filtered) was added to each tube. The tubes were again covered with foil and placed into a boiling water bath for exactly seven minutes. Once cooled the

absorbance was measured at 830 nm using a Bosch and Lomb Colorimeter. The blank consisted of perchloric acid, water, ammonium molybdate and ANSA.

2.2.5 Thin Layer Chromatography

Phospholipids were separated by thin layer chromatography (TLC) on silica H plates that had been activated by placing in an oven at 110°C for one hour. Filter paper was added to the TLC chamber and allowed to equilibrate for 30 minutes with the solvent system. The solvent system consisted of chloroform, methanol, 2–propanol, triethylamine (added slowly) and 0.25% (w/v) KCl (15:4.5:12.5:9:3) [90]. The KCl was added dropwise. From the original lipid sample 100μl was dried under nitrogen and resuspended in 50μl hexane. Sample was spotted on silica H plates, the tube was washed with 50μl hexane and this was spotted over the original spot. The plate was dried and inserted into the TLC chamber for 80–90 minutes. After development the plate was air dried in the fumehood, sprayed lightly with 0.1% (w/v) filtered ANSA (8–anilino–1–naphthalene sulfonic acid in milliQ water) and viewed under UV light [84]. The phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) spots were marked, scraped off the plate and extracted with 10 mls chloroform:methanol (1:1 with 50 mg/L BHT). Five mls of milliQ water were added, the tubes vortexed 20–30 seconds and the layers allowed to separate in the refrigerator. The chloroform layer was collected, dried, resuspended in 500μl hexane and the phosphorus analysed as previously described [57]. Samples were stored at -70°C until required for alkenyl and diacyl analysis.

2.2.6 Determination of Alkenyl and Diacyl Groups

PC and PE samples were benzoylated to determine the alkenyl and diacyl content of each fraction by HPLC [4][61]. The amount of sample benzoylated varied between 50 and 200μg. The appropriate amount of sample was dried under nitrogen and redissolved in four mls of diethylether. To each sample was added one ml of borate buffer 0.49% (w/v) boric acid, 11.69% (w/v) sodium chloride, 0.32% (w/v) calcium chloride, pH 8) which contained one μl phospholipase C (PLC) [61]. The PLC was type XI from B. cereus. The tubes were shaken in a water bath for one hour at 37°C. The top (diethylether) layer was collected, transferred to a 13 X 100 mm extraction tube and dried under nitrogen. Then 200μl of 5% (w/v) benzoic anhydride in benzene and 100μl of 2% (w/v) 4–dimethylaminopyridine in benzene were added to each tube, mixed and allowed to stand at room temperature for one hour [4]. The tubes were then placed on ice for 15–30 minutes and 2 mls of 0.1N NaOH were added. Ice was used to prevent saponification [4]. This solution was extracted three times with hexane, dried under nitrogen and dissolved in 100μls chloroform. The chloroform solution was applied to a 100–200 mesh silicic acid column (Unisil) and eluted with 11–12 mls HPLC grade chloroform. The chloroform was dried under nitrogen, the tube washed with three mls chloroform, dried again and then resuspended in 200μls HPLC grade cyclohexane. Samples were stored at -70°C until run on the HPLC machine. Separation of lipid classes was done on a Waters HPLC using a Nova Pak Silica column 3.9 X 150 mm. Solvent flow rates were 1.3 ml/min and detection of resolved components was made at 230 nm.

2.2.7 Fatty Acid Analysis of the Alkenyl and Diacyl Subclass of the PC Fraction From The Distal Small Intestine and The PE Fraction From The Proximal Small Intestine

R_f values for standard alkenyl and diacyl fractions were determined on silica G plates using benzene/hexane/diethylether (50:45:4, by volume) as the mobile phase. The R_f values are 0.69 and 0.32 respectively (see Figure 2.1). Silica G plates were activated at 110°C for one hour. Plates were spotted with 100μl benzoylated lipid extract and developed for 35 minutes in a tank containing HPLC grade benzene/HPLC grade hexane/HPLC grade diethylether (50:45:4, by volume). Plates were air dried and sprayed with 0.1% (w/v) 8–anilino–1–naphthalene sulfonic acid. The spots corresponding to the standard alkenyl and diacyl R_f values were scraped into methylation tubes and methylated according to the method by Morrison [59]. Before methylation 10μg of C19 standard was placed into each tube. Samples were reconstituted in one ml redistilled hexane for GC on a Varian GC6000 gas chromatograph machine equipped with a DS 654 data system. A BP20 polyethylene glycol (PEG) fused silica polar capillary column with a column lengh of 25m and an internal diameter of 0.22mm was used [39]. The carrier gas was helium, the split ratio used was 1:100 and the flowrate was 1.5 ml/min. The fatty acid composition of the alkenyl and diacyl subclasses of the PC fraction from the distal small intestinal mucosa and the PE fraction from the proximal small intestinal mucosa can be found in Tables 3.9, 3.10, 3.11 and 3.12, respectively. Gas chromatography was used to measure fatty acids from C14:0 to C22:6(3).

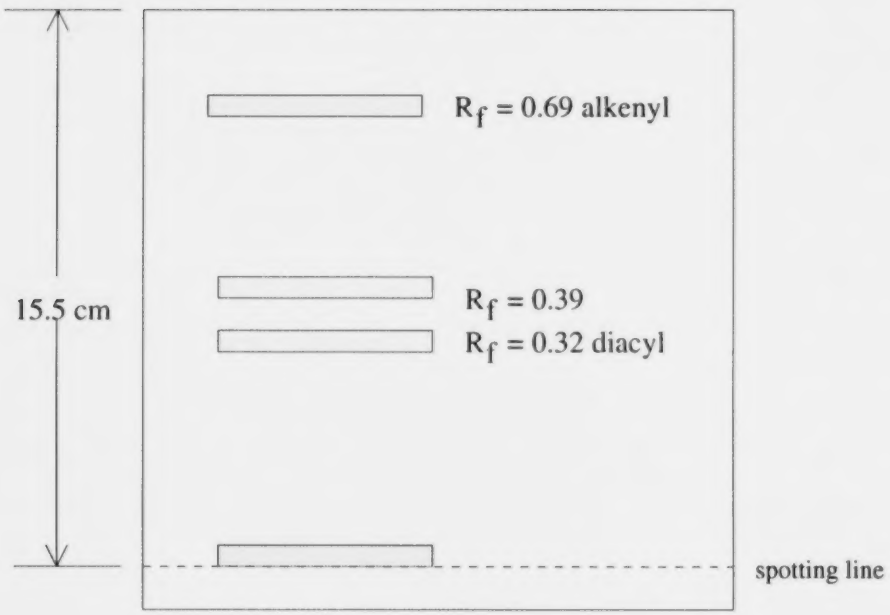

Figure 2.1: The pattern of alkenyl and diacyl subclass of PE and PC fractions on a Silica G plate. The solvent system used was benzene/hexane/diethylether (50:45:4, by volume.)

2.2.8 Statistics

The mean and standard error of the mean (SEM) for the rat weights, amount of food consumed, total amount of phospholipid in each tissue, content of PC and PE and the relative percentage of alkenyl and diacyl fraction is indicated for each diet group. The effect of diet treatment on alkenyl and diacyl content of PE and PC was examined by a one–way ANOVA using SPSS and significant differences between diet groups were found by the multiple range test Student-Newman-Kuels (SNK) and by contrast tests on SPSS. A one–way ANOVA was also performed on the fatty acid data and significant differences between diet groups were found by the multiple range test SNK and by contrast tests on SPSS.

Chapter 3

Results and Discussion

3.1 Results

3.1.1 Diet

The components of the four experimental diets are shown in Table 2.1 and the fatty acid composition of each diet can be found in Table 3.1. Each diet contains adequate C18:2(6) to prevent an essential fatty acid deficiency [35]. The safflower oil (SFO) diet contained a significant amount of linoleic acid (74.6%) and approximately half the oleic acid contained in the T&SFO and the T&SFO&DHA or EPA diets. Each diet contained approximately equal amounts of α–linolenic acid and only the SFO diet showed a significantly different linoleic acid to saturated fat ratio (7.7 vs an average of 0.23). All diets except the SFO diet contained approximately equal amounts of saturated fat (average 44.5%).

3.1.2 Body Weights and Food Consumption

All rats appeared to be healthy after four weeks of diet treatment. Even though there were small but significant differences in body weights between dietary groups at the beginning of the feed period, after four weeks all groups weighed approximately the same amount (see Table 3.2). The fish oil diets produced the heaviest rats while the lightest rats were from the T&SFO diet but the differences were not significant. The differences in weight found at the beginning of the experiment may be due to the

fact that the rats were started on their diets in a staggered fashion and were weighed immediately before the test period.

There was no significant difference in the amount of food that each group consumed in weeks one and four however the rats from the SFO diet ate the least amount during both weeks (see Table 3.4).

3.1.3 Diet Treatment Effect on Mucosal Tissue Weights

The EPA dietary group produced the lowest mucosal weight in the proximal and distal small intestine but the differences between groups was not significant. In the proximal small intestine the average wet weight of the mucosa from the EPA dietary group was 0.9±0.0 grams and from the other three dietary groups it was averaged to be 1.1±0.1 grams. In the distal small intestine the average wet weight of the mucosa from the EPA group was 0.6±0.1 grams and from the other three dietary groups it was averaged to be 0.7±0.0 grams (see Table 3.3). By contrast, the EPA dietary group produced the heaviest colon while the lightest colon came from the SFO dietary group. These results cannot be explained by differences in food consumption or body weights between groups because there were no significant differences in the amount of food consumed or in the weight of each group before sacrifice (see Tables 3.2 and 3.4).

Weight of mucosa obtained from the proximal small intestine was greater than that obtained from the distal small intestine.

3.1.4 Diet Treatment and Phospholipid Composition of the Mucosal Cells.

Diet fat fed did not significantly effect the total phospholipid content of the mucosal cells in the proximal and distal small intestine or in the colon. Generally, the amount of phospholipid produced was in the following order: colon > proximal small intestine > distal small intestine. The SFO diet produced the least amount of total phospholipid regardless of the tissue examined (proximal total phospholipid was 1.3±0.2 mg/g wet tissue weight; distal total phospholipid was 1.0±0.2 mg/g wet tissue weight; colon total phospholipid was 1.4±0.2 mg/g wet tissue weight). In all three sections of the intestine more PC than PE was found in all four dietary groups (see Tables 3.5 and 3.6). In the proximal small intestine and in the colon the tissue obtained from the T&SFO diet group produced significantly more PC than did the tissue obtained from the other three dietary groups (see Table 3.6). No such difference was found for the distal small intestine.

Table 3.1: Fatty acid composition of experimental diets. The fatty acid composition is T = beef tallow; SFO = safflower oil; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid.

Fatty acid	*Diet fat fed*			
	T&SFO	SFO	T&SFO &DHA	T&SFO &EPA
14:0	3.3	-	3.8	4.0
16:0	27.2	7.2	26.2	23.0
16:1(n-7)	3.2	-	3.6	4.6
18:0	17.7	2.5	14.2	14.1
18:1(n-9)	38.6	15.5	33.6	33.1
18:2(n-6)	9.7	74.6	10.4	10.0
18:3(n-3)	0.3	0.3	0.4	0.4
20:4(n-6)	-	-	0.4	0.3
20:5(n-3)	-	-	1.4	6.6
22:5(n-3)	-	-	0.4	0.7
22:6(n-3)	-	-	5.6	3.2
P/S ratio	0.2	7.7	0.4	0.5
Σsat	48.2	9.7	44.2	41.1
ΣPUFA	10	74.9	18.6	21.2
ΣMUFA	41.8	15.5	37.2	37.7
UI[a]	60	170	100	120
Σn-9	38.6	15.5	33.6	33.1
Σn-6	9.7	74.6	10.8	10.3
Σn-3	0.3	0.3	7.8	10.9
n6/n3	32.3	249	1.4	1.0
C18:2(6)/Σsat	0.2	7.7	0.2	0.2

[a]UI = Σ([a][b]), where 'a' is the percent of each unsaturated fatty acid and 'b' is the number of double bonds for that particular fatty acid.

Table 3.2: Average body weights of the rats in each group. Represented as the amount (g) ± the standard error of the mean. The average weights of the rat groups before the experiment started is shown as week 0. Weeks 1,2 and 3 show the average weights after the respective week and week 4 is the average weight of the groups just before sacrifice. Numbers within a column with different superscripts are significantly different ($p<0.05$). Numbers within brackets indicate the number of rats in each group.

Diet		*Week*				
		0	1	2	3	4
T&SFO	(6)	55.7 ± 0.2^a	132.0±10.3	195.4±11.6	259.4±11.4	319.4±10.0
SFO	(7)	53.0 ± 0.8^a	141.9±11.6	191.7±8.7	258.8±8.5	330.0±9.6
T&SFO&DHA	(7)	60.4 ± 0.4^b	135.2±8.0	199.5±10.6	263.7±16.0	335.8±16.1
T&SFO&EPA	(6)	57.8 ± 0.3^c	138.1±12.1	204.2±15.6	273.2±15.9	341.8±15.3

Table 3.3: Average wet weight of mucosa from the proximal and distal small intestine and the average wet weight of the colon. Data is given for all four dietary groups and is in grams (±SEM). T&SFO = tallow and safflower oil; SFO = safflower oil; T&SFO&DHA = tallow, safflower oil and docosahexaenoic acid; T&SFO&EPA = tallow, safflower oil and eicosapentaenoic acid. No significant differences were found between groups ($p<0.05$).

Diet	*Tissue*		
	Proximal	Distal	Colon
	grams	grams	grams
T&SFO	1.1±0.1	0.8±0.1	1.2±0.1
SFO	1.0±0.1	0.8±0.0	1.1±0.1
T&SFO&DHA	1.1±0.2	0.6±0.0	1.2±0.1
T&SFO&EPA	0.9±0.0	0.6±0.1	1.4±0.0

Table 3.4: Average amount of food eaten by each rat in each group of rats during week one and week four. Data is given in grams ± standard error of the mean. No significant differences were found between groups ($p<0.05$).

Diet	*Week*	
	1	4
T&SFO	107.5±7.2	156.7±7.1
SFO	95.2±3.9	154.1±6.7
T&SFO&DHA	103.9±4.8	165.2±4.8
T&SFO&EPA	107.3±9.1	154.9±9.4

Table 3.5: Average total amount of phospholipid and PE found in two sections of the small intestine and in the colon of four groups of rats each fed one of four different diets for four weeks. Data is given in mg/g wet tissue ± SEM. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow, safflower oil and docosahexaenoic acid diet; T&SFO&EPA = tallow, safflower oil diet and eicosapentaenoic acid diet; pL = phospholipid. Numbers within a column with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Diet		*Tissue*					
		Proximal		*Distal*		*Colon*	
		Total pL mg/g	PE mg/g	Total pL mg/g	PE mg/g	Total pL mg/g	PE mg/g
T&SFO	(6)	1.8±0.3	0.2±0.1	1.0±0.2	0.2±0.1	2.6±0.5	0.2±0.0
SFO	(7)	1.3±0.2	0.1±0.1	1.0±0.2	0.2±0.1	1.4±0.2	0.1±0.0
T&SFO&DHA	(7)	1.9±0.4	0.2±0.0	1.5±0.3	0.2±0.0	1.6±0.2	0.1±0.0
T&SFO&EPA	(6)	1.6±0.2	0.2±0.1	1.6±0.2	0.2±0.1	1.9±0.2	0.2±0.0

Table 3.6: Average total amount of phospholipid and PC found in the mucosa of two sections of the small intestine and in the colon of four groups of rats each fed one of four different diets for four weeks. Data is given in mg/g wet tissue ±SEM. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow, safflower oil and docosahexaenoic acid diet; T&SFO&EPA = tallow, safflower oil and eicosapentaenoic acid diet; pL = phospholipid. Numbers within a column with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Diet		*Tissue*					
		Proximal		*Distal*		*Colon*	
		Total pL mg/g	PC mg/g	Total pL mg/g	PC mg/g	Total pL mg/g	PC mg/g
T&SFO	(6)	1.8±0.3	0.6 ± 0.2^{a}	1.0±0.2	0.3±0.07	2.6±0.5	0.6 ± 0.1^{a}
SFO	(7)	1.3±0.2	0.2 ± 0.1^{b}	1.0±0.2	0.2±0.1	1.4±0.2	0.2 ± 0.04^{b}
T&SFO&DHA	(7)	1.9±0.4	0.3 ± 0.1^{b}	1.5±0.3	0.4±0.1	1.6±0.2	0.2 ± 0.1^{b}
T&SFO&EPA	(6)	1.6±0.2	0.3 ± 0.1^{b}	1.6±0.2	0.3±0.0	1.9±0.2	0.2 ± 0.2^{b}

Diet had no significant effect on the amount PE produced in any of the tissue examined. As a percentage, the distal small intestine produced more PE than did the proximal small intestine or the colon (16% compared to 10% and 8% respectively). This was also true for the PC content of the total phospholipid (on average 24% compared to 21% and 15% respectively).

3.1.5 Effect of Diet on Alkenyl and Diacyl Subclasses of the PE and PC Fraction of the Mucosal Cells

In general, in all three sections of the intestine and in all four dietary groups more of the alkenyl subclass of the PC and PE fraction was found than the diacyl (see Tables 2.8 and 2.9). In the proximal small intestine there was a significant difference between the two fish oil diets in the relative amount of alkenyl and diacyl subclasses of PE. The EPA dietary group had more alkenyl content (93.4±1.4%) and less diacyl content (6.7±1.4%) than did the DHA group (72.3±5.8% and 27.7±5.8%). The SFO dietary group produced more of the alkenyl subclass (85.4±3.6%) and less of the diacyl subclass (14.6±3.6%) than did the DHA dietary group while the T & SFO dietary group produced significantly less of the alkenyl group and more of the diacyl group than did the EPA dietary group (see Table 2.8). No significant differences were found in the alkenyl and diacyl content of the PE fraction between dietary groups in the distal small intestine or in the colon.

Table 3.7: Relative (%±SEM) and absolute (mg/g wet tissue weight±SEM) of the alkenyl and diacyl subclasses of the PE fraction of total phospholipid from the proximal and distal small intestine and from the colon. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow, safflower oil and docosahexaenoic acid diet; T&SFO&EPA = tallow, safflower oil and eicosapentaenoic acid diet. Numbers within a column within a tissue class with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Tissue	*Diet*		*Lipid Subclass*			
			Alkenyl		*Diacyl*	
			(%)	mg/g	(%)	mg/g
Proximal	T&SFO	(6)	79.9 ± 5.9^{ac}	0.2 ± 0.1	20.1 ± 5.9^{ac}	0.05 ± 0.02^{abc}
	SFO	(7)	85.4 ± 3.6^{ab}	0.1 ± 0.0	14.6 ± 3.6^{ab}	0.01 ± 0.0^{a}
	T&SFO&DHA	(7)	72.3 ± 5.8^{c}	0.1 ± 0.0	27.7 ± 5.8^{c}	0.05 ± 0.01^{b}
	T&SFO&EPA	(6)	93.4 ± 1.4^{b}	0.2 ± 0.1	6.7 ± 1.4^{b}	0.01 ± 0.0^{ac}
Distal	T&SFO	(6)	79.9 ± 4.3	0.2 ± 0.1	20.1 ± 4.3	0.05 ± 0.02
	SFO	(7)	77.2 ± 3.6	0.1 ± 0.1	22.8 ± 3.6	0.04 ± 0.02
	T&SFO&DHA	(7)	79.0 ± 3.1	0.2 ± 0.0	21.0 ± 3.1	0.04 ± 0.01
	T&SFO&EPA	(6)	79.7 ± 4.1	0.2 ± 0.1	20.3 ± 4.1	0.04 ± 0.01
Colon	T&SFO	(6)	75.2 ± 4.0	0.1 ± 0.0	24.8 ± 4.0	0.05 ± 0.01
	SFO	(7)	80.3 ± 4.5	0.1 ± 0.0	19.7 ± 4.5	0.02 ± 0.01
	T&SFO&DHA	(7)	77.3 ± 2.2	0.1 ± 0.0	22.7 ± 2.2	0.03 ± 0.01
	T&SFO&EPA	(6)	82.4 ± 1.6	0.1 ± 0.0	17.6 ± 1.6	0.03 ± 0.0

Table 3.8: Relative (%±SEM) and absolute (mg/g wet tissue weight±SEM) of the alkenyl and diacyl subclasses of the PC fraction of total phospholipid from the proximal and distal small intestine and from the colon. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow, safflower oil and docosahexaenoic acid diet; T&SFO&EPA = tallow, safflower oil and eicosapentaenoic acid diet. Numbers within a column within a tissue class with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Tissue	*Diet*		*Lipid Subclass*			
			Alkenyl		*Diacyl*	
			(%)	mg/g	(%)	mg/g
Proximal	T&SFO	(6)	64.6±4.5	0.4±0.1	35.4±4.5	0.21±0.06
	SFO	(7)	67.8±7.3	0.1±0.0	32.2±7.3	0.10±0.03
	T SFO&DHA	(7)	67.0±7.3	0.2±0.0	33.0±7.3	0.13±0.06
	T&SFO&EPA	(6)	67.3±9.3	0.2±0.1	32.7±9.3	0.10±0.06
Distal	T&SFO	(6)	82.2 ± 3.2^{a}	0.2 ± 0.1^{a}	17.8 ± 3.2^{a}	0.05 ± 0.01^{b}
	SFO	(7)	62.9 ± 7.2^{bcd}	0.1 ± 0.0^{b}	37.1 ± 7.2^{b}	0.10 ± 0.05^{ab}
	T&SFO&DHA	(7)	51.8 ± 3.1^{c}	0.2 ± 0.1^{ab}	48.2 ± 3.1^{b}	0.18 ± 0.04^{a}
	T&SFO&EPA	(6)	64.3 ± 3.3^{d}	0.2 ± 0.0^{ab}	35.6 ± 3.3^{b}	0.12 ± 0.02^{a}
Colon	T&SFO	(6)	69.4±4.5	0.4 ± 0.1^{a}	30.6±4.5	0.18 ± 0.04^{a}
	SFO	(7)	68.0±4.8	0.1 ± 0.0^{b}	32.0±4.8	0.06 ± 0.01^{b}
	T&SFO&DHA	(7)	72.0±3.6	0.2 ± 0.0^{b}	28.0±3.6	0.06 ± 0.01^{b}
	T&SFO&EPA	(6)	78.3±5.8	0.2 ± 0.0^{ab}	21.7±5.8	0.05 ± 0.01^{b}

Diet had a significant effect in the alkenyl and diacyl subclasses of the PC fraction in the distal small intestine (see Table 3.9). There was a significant increase in the alkenyl subclass in the T&SFO diet group compared to the SFO, T&SFO&DHA and T&SFO&EPA diet groups. The relevant values are 82.2±3.2%, 62.9±7.2%, 51.8±3.1% and 64.3±3.3% respectively. The animals fed DHA produced a significant reduction in the alkenyl subclass of PC. No significant differences were found in the proximal small intestine. If only relative amounts (%) are considered then dietary treatment had no effect on the alkenyl and diacyl contents in the colon. However, if absolute amounts (mg/g wet tissue weight) are considered then significantly more alkenyl and diacyl lipids were found in the colon of the T&SFO dietary groups compared to the other three dietary groups. There was a four fold increase in the alkenyl subclass in the T&SFO dietary group compared to the SFO group and there was a two fold increase compared to the fish oil diet groups. Approximately three times as much diacyl subclass was produced in animals fed the T&SFO diet compared to those fed the other three diets.

3.1.6 Fatty Acid Composition of the Alkenyl and Diacyl Subclass of PE and PC

Alkenyl Subclass of the PC Fraction in the Distal Small Intestine

The major fatty acids in the alkenyl subclass of the PC fraction of the distal small intestine were C16:0, C18:0 and C18:1(9&7), representing 18.2%, 26.9% and 6.0% respectively. This accounts for more than 50% of the total fatty acid content (see Table 3.10). Groups fed highly unsaturated fatty acid diets (high unsaturation index (UI)) increased the UI index in the tissue and decreased the C16:0 fatty acid content.

Generally, as the UI of the diet decreased so did the UI of the tissue (see Tables 3.1 and 3.9).

Table 3.9: Fatty acid distribution of alkenyl phosphatidylcholine from the distal small intestine. Data is given as mean (±SEM) on purified alkenyl fractions. Data is given as area% rather than mol%. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow and safflower oil and docosahexaenoic acid diet; T&SFO&EPA = tallow and safflower oil and eicosapentaenoic acid diet. Numbers within a row with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Fatty Acid	*Diet Fat Fed*			
Fatty Acid	T & SFO	SFO	T & SFO & DHA	T & SFO & EPA
	(6)	(7)	(7)	(6)
16:0	18.2±3.4	10.3±3.6	11.9±2.5	13.3±1.4
16:1(5)	1.0±0.4	1.6±0.5	0.8±0.4	1.3±0.4
18:0	26.9±2.3^{a}	23.0±2.2^{ab}	25.4±2.6^{ab}	17.4±0.6^{b}
18:1(9&7)	6.0±0.7	5.2±0.9	6.4±0.6	6.4±0.8
18:2(6)	1.7±0.4	0.8±0.4	1.7±0.4	1.3±0.2
18:3(6)	2.4±1.0^{a}	2.0±0.7^{a}	2.3±0.8^{a}	6.7±1.2^{b}
18:3(3)	2.5±0.5^{a}	2.5±0.6^{a}	2.3±0.6^{a}	4.3±0.7^{b}
18:4(3)	2.3±0.8	3.2±1.2	3.0±0.9	3.0±0.6
20:3(9)	3.2±0.7	3.3±0.5	2.4±0.9	7.3±2.0
20:3(6)	1.6±1.2	3.7±1.4	4.8±1.2	4.0±0.9
20:4(6)	4.0±1.3^{ab}	4.5±1.4^{a}	1.1±0.4^{b}	1.6±0.6^{ab}
20:5(3)	1.8±0.6	1.0±0.5	1.2±0.7	4.1±1.5
22:4(6)	3.3±0.9	10.3±5.0	10.0±4.3	2.6±0.8
22:5(6)	0.9±0.2^{ab}	0.7±0.3^{a}	0.5±0.3^{a}	1.6±1.0^{b}
22:5(3)	1.4±0.6^{a}	1.1±0.4^{a}	4.0±1.1^{b}	4.9±1.1^{b}
22:6(3)	3.4±0.9	5.2±1.4	2.4±0.6	1.9±0.6
Σ saturates[a]	59.1	47.3	49.7	41.3
Σ PUFA	28.5	38.3	35.7	43.3
Σ MUFA	13.3	13.8	14.3	13.8
UI[b]	120.5	167.1	153.2	176.5
Σ n-9[c]	7.7	13.8	13.7	18.0
Σ n-6	13.9	22.0	20.4	17.8
Σ n-3	11.4	13.0	12.9	18.2
Σ(n6/n3)	1.2	1.7	1.6	1.0

[a]Includes fatty acids from C14:0 to C22:6(3).

[b]UI = Σ([a][b]), where 'a' is the percent of each unsaturated fatty acid and 'b' is the number of double bonds for that particular fatty acid

[c]Includes C18:1(7).

Diet content of C16:0 had no significant effect on alkenyl content of C16:0 in the mucosa of the distal small intestine. Slightly more C16:0 was obtained from the dietary group eating the greatest amount of palmitic acid when compared to the other three dietary groups. The T&SFO dietary group also produced the greatest amount of C18:0 compared to the SFO, DHA, and the EPA dietary groups but it was only significant when compared to the EPA dietary group (26.9% vs 17.4%). Diet had no significant effect on the tissue content of C18:1(9).

Oddly enough the group fed the diet highest in C18:2(6) (the SFO group) produced a decrease in the alkenyl C18:2(6) compared to the T&SFO group but the change was not significant. A significant increase in alkenyl C18:3(6) was found in the EPA dietary group when compared to the other three dietary groups (see Table 3.9) and yet the EPA group had very low C20:4(6) content in their alkenyl subclass. In contrast, the DHA group had a low amount of C18:3(6) in their alkenyl subclass and the lowest C20:4(6), significantly lower than the SFO and slightly lower than the T&SFO diet groups (see Figure 3.1). Diet had no significant effect on the level of EPA or DHA in the tissue; slightly more C20:5(3) was obtained from the EPA dietary group.

Diet significantly effected the level of C20:4(6) in the alkenyl subclass of PC (see Figure 3.2). The lowest levels of alkenyl C20:4(6) were observed in the animals fed fish oil. Only the DHA diet caused a significant reduction in C20:4(6). It should be noted that the DHA diet also caused a significant reduction in the amount of alkenyl subclass produced compared to the EPA and T&SFO diets but not compared to the SFO diet.

The EPA dietary group had significantly more C22:5(6) in their alkenyl subclass

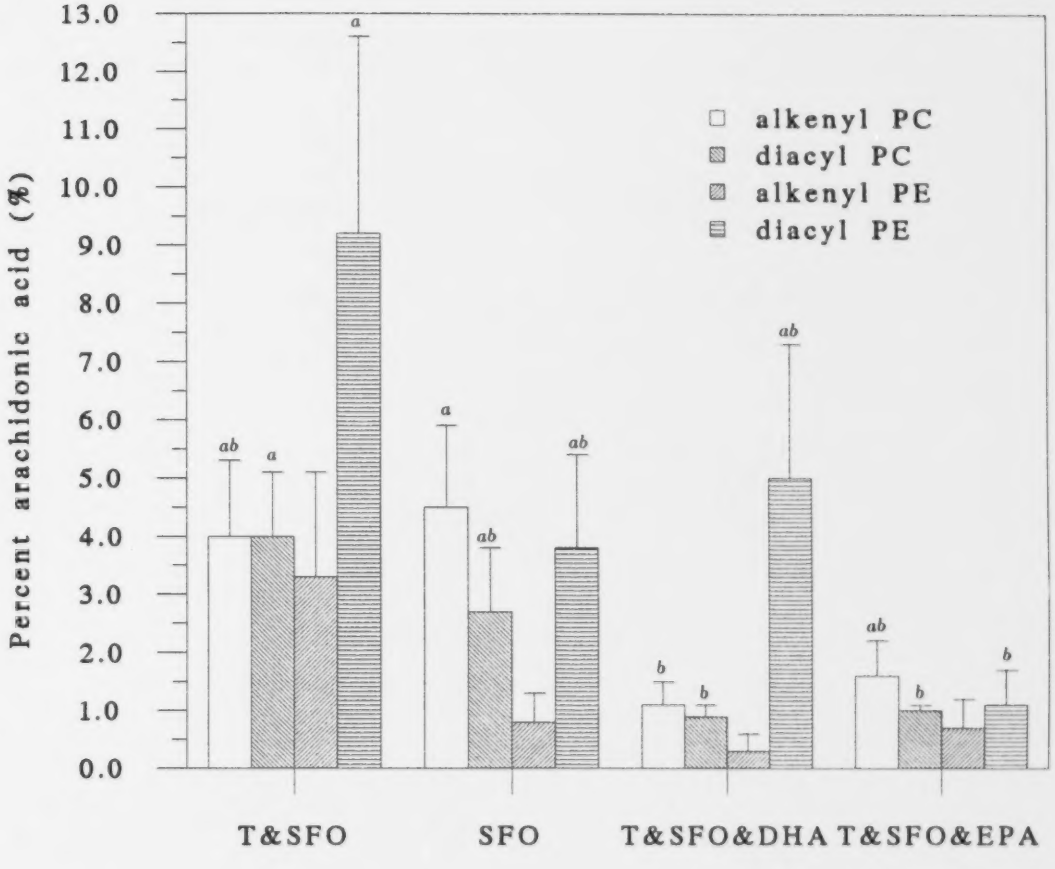

Figure 3.1: The effect of dietary fatty acid treatment on the arachidonic acid content of the alkenyl and diacyl subclasses of the PC fraction from the mucosa of the distal small intestine and of the PE fraction from the mucosa of the proximal small intestine of the rat. T&SFO=tallow and safflower oil diet; SFO=safflower oil diet; T&SFO&DHA=tallow, safflower and docosahexaenoic acid diet; T&SFO&EPA=tallow, safflower and eicosapentaenoic acid diets. Columns of the same coding but with different superscripts are significantly different ($p<0.05$)

of PC than the SFO and T&SFO&DHA groups. It also had significantly more C22:5(3) than the SFO and T&SFO diet groups but not more than the DHA diet group. It is interesting to note that although the DHA and EPA diets produced high levels of C22:5(3) (4.0±1.1% and 4.9±1.1% respectively) they had very low levels of C22:6(3) (2.4±0.6% and 1.9±0.6% respectively).

Finally, there seemed to be a build up of Mead acid in the alkenyl subclass from the EPA dietary group compared to the T&SFO, SFO and T&SFO&DHA groups.

Diacyl Subclass of the PC Fraction in the Distal Small Intestine

Diet effected the major fatty acyl constituents of the diacyl subclass of PC in the distal small intestine (see Table 3.10). There was a significant decrease in C22:5(3), C20:5(3) which accounted for the lower total n3 fatty acyl content of the diacyl sublass of PC in the group fed the T&SFO diet compared to the other three groups.

A significant decrease in C16:0 content of the PC fraction accounts for the reduced total saturated fatty acid content in the SFO diet compared to the other three diets. The SFO group also had the largest n9 fatty acid content. This was mostly due to the increase in C20:1(9) and C22:1(9). The highest polyunsaturated fatty acid to saturated fatty acid ratio (P/S ratio) was also found in the SFO dietary group and was due to the increase in C18:4(3), C22:5(3) and C22:6(3) as well as the decrease in C16:0.

Diet had a significant effect on C20:4(6) levels in the diacyl subclass of PC from the distal small intestine. There was a significant reduction in C20:4(6) content in the groups fed DHA or EPA compared to the T&SFO diets (see Table 3.10 and Figure 3.1). It should be noted that the animals fed fish oil produced significantly

lower levels of alkenyl PC compared to the T&SFO group.

As was found in the alkenyl group there was a significant increase in Mead acid in the diacyl subclass in the rats fed EPA. In contrast to the alkenyl subclass there was also an increase in Mead acid in the SFO group. The EPA and DHA groups showed a significant increase in the C22:5(3) content compared to the T&SFO group without a concomitant increase in C22:6(3).

Table 3.10: Fatty acid distribution of diacyl phosphatidylcholine from the distal small intestine. Data is given as mean (± SEM) on purified diacyl fractions. Data is given as area % rather than mol %. T&SFO = tallow and safflower oil diet; SFO = safflower oil diet; T&SFO&DHA = tallow and safflower and docosahexaenoic acid diet; T&SFO&EPA = tallow and safflower and eicosapentaenoic acid. Numbers within a row with different superscripts are significantly different ($p<0.05$). Numbers in brackets indicate the number of rats in each group.

Fatty Acid	*Diet Fat Fed*			
Fatty acid	T&SFO	SFO	T&SFO &DHA	T&SFO &EPA
	(6)	(7)	(7)	(6)
16:0	22.6 ± 5.3^{a}	5.4 ± 1.6^{b}	18.6 ± 2.8^{a}	21.2 ± 2.2^{a}
16:1(5)	0.5±0.2	0.5±0.3	0.9±0.2	0.7±0.2
18:0	22.7 ± 2.4^{ab}	18.0 ± 1.7^{a}	27.7 ± 1.8^{b}	20.8 ± 0.9^{a}
18:1(9&7)	10.0 ± 0.6^{a}	4.4 ± 0.6^{b}	12.8 ± 1.9^{a}	8.6 ± 1.1^{ab}
18:2(6)	5.4±1.6	3.2±1.0	5.6±1.6	3.2±0.4
18:3(6)	0.6±0.4	2.6±0.9	2.5±1.2	2.2±1.2
18:3(3)	2.4±1.0	2.5±0.5	2.4±0.6	2.2±0.3
18:4(3)	0.7±0.4	2.7±0.8	1.4±0.4	0.8±0.2
20:3(9)	0.7 ± 0.2^{a}	5.4 ± 2.0^{ab}	1.5 ± 0.4^{a}	6.5 ± 1.7^{b}
20:3(6)	2.4±1.5	5.0±1.6	3.2±0.6	2.7±0.7
20:4(6)	4.0 ± 1.1^{a}	2.7 ± 1.1^{ab}	0.9 ± 0.2^{b}	1.0 ± 0.1^{b}
20:5(3)	0.0 ± 0.0^{a}	1.9 ± 0.8^{ab}	0.9 ± 0.3^{b}	3.4 ± 1.1^{b}
22:4(6)	7.8±4.1	9.4±7.3	5.0±1.5	9.7±4.0
22:5(6)	0.4 ± 0.2^{ab}	0.9 ± 0.4^{ab}	0.1 ± 0.1^{a}	2.8 ± 1.0^{b}
22:5(3)	0.0 ± 0.0^{a}	4.0 ± 1.7^{ab}	2.5 ± 0.6^{b}	2.7 ± 0.5^{b}
22:6(3)	2.7±0.9	4.1±1.6	1.3±0.5	1.0±0.2
Σ saturates[a]	56.7	32.9	54.8	47.8
Σ PUFA	27.1	44.4	27.3	39.3
Σ MUFA	15.9	15.4	17.1	12.4
UI[b]	113.2	186.1	111.6	159.4
Σ n-9[c]	15.0	19.3	16.3	17.3
Σ n-6	20.6	23.8	17.3	22.7
Σ n-3	5.8	15.2	8.5	10.1
Σ(n6/n3)	3.6	1.6	2.0	2.2

[a]Includes fatty acids from C14:0 to C22:6(3).

[b]UI=Σ([a][b]), where 'a' is the percent of each unsaturated fatty acid and 'b' is the number of double bonds for that particular fatty acid.

[c]Includes C18:1(7).

3.1.7 Alkenyl Subclass of the PE Fraction in the Proximal Small Intestine

Approximately half the fatty acid composition of the alkenyl subclass was from C16:0, C18:0, C18:1(9&7) (see Table 3.11). There appears to be no correlation between the degree of unsaturation of the diet and the unsaturation index of the tissue.

Diet had very little effect on the fatty acid composition of the alkenyl subclass of PE obtained from the proximal small intestine. The increase in monounsaturated fat level in the animals fed EPA can be accounted for by the increase in C14:1(7), C18:1(7) and C20:1(9). The increase in n-9 fatty acids in the animals fed T&SFO is due to the increase in C18:1(9), and the decrease in n-3 fatty acids in the EPA dietary group is due to the decrease in C20:5(3), C22:5(3) and C22:6(3).

The level of C18:2(6) was significantly increased in the animals fed the EPA diet compared to those fed DHA but not compared to those fed SFO or T&SFO.

Diet had no significant effect on C20:4(6) level in the alkenyl subclass of PE (see Table 3.11 and Figure 3.1). The C20:4(6) data is difficult to assess due to the extent of the error obtained, however it is interesting to note that although the EPA diet caused a reduction in tissue C20:4(6), it also caused a significant increase in the amount of ether phospholipid produced compared to the DHA and T&SFO diet but not compared to the SFO diet. On the other hand, animals fed DHA produced the least amount of C20:4(6) in the alkenyl subclass of PE and they also produced a significant reduction in alkenyl levels when compared to the EPA and SFO diet but not compared to the T&SFO diet.

3.1.8 Diacyl Subclass of the PE Fraction in the Proximal Small Intestine

Diet effected fatty acid composition of the diacyl subclass of PE more than it did the alkenyl subclass. The increase in n-9 content in the animals fed DHA and EPA is accounted for by the increase in C18:1(9), C20:1(9) and C22:1(9). The increase in n-6 in the animals fed T&SFO is due to an increase in C18:2(6) and C20:4(6).

The EPA diet caused a significant increase in C16:0 compared to the other three diets. The animals fed DHA produced more C18:0 compared to those fed EPA. The DHA diet also caused a significant reduction in C17:0 compared to the SFO diet (see Table 3.12).

Diet had an interesting effect on diacyl C20:4(6) content. The EPA diet caused a significant reduction in the level of C20:4(6) compared to the T&SFO diet but the DHA diet did not have a similar effect. The animals fed EPA produced the least amount of C20:4(6) in the diacyl fraction of PE and they also produced a significant decrease in the diacyl levels of PE.

Table 3.11: Fatty acid distribution of alkenyl phosphatidylethanolamine from the proximal small intestine. Data is given as mean (±SEM) on purified alkenyl fractions. Data is given as area% rather than mol%. T & SFO = tallow and safflower oil diet; SFO = safflower oil diet; T & SFO & DHA = tallow and safflower oil and docosahexaenoic acid diet; T & SFO & EPA = tallow and safflower oil and eicosapentaenoic acid diet. Numbers within a row with different superscripts are significantly different ($p<0.05$). Numbers within the brackets indicate the number of rats in each group.

Fatty Acid	*Diet Fat Fed*			
Fatty Acid	T & SFO (6)	SFO (6)	T & SFO & DHA (7)	T & SFO & EPA (5)
16:0	11.4±4.2^{ab}	12.6±6.0^{ab}	3.6±2.1^{a}	16.6±2.6^{b}
16:1(5)	0.0±0.0	0.2±0.2	0.0±0.0	0.5±0.3
18:0	26.1±7.5	23.1±3.6	20.3±5.3	24.2±2.9
18:1(9&7)	10.5±4.7	4.6±1.7	2.0±1.4	5.1±1.7
18:2(6)	1.3±0.7^{ab}	1.8±0.8^{ab}	0.3±0.3^{a}	3.4±1.1^{b}
18:3(6)	4.0±0.9	5.4±2.1	6.4±1.5	2.0±0.7
18:3(3)	2.0±1.2	2.8±1.8	0.0±0.0	0.4±0.4
18:4(3)	0.3±0.3	0.7±0.7	0.6±0.6	0.4±0.2
20:3(9)	4.1±0.9^{a}	1.4±0.7^{b}	5.7±3.5^{ab}	4.0 ± 2.0^{ab}
20:3(6)	3.2±1.4	7.7±2.4	5.5±2.2	5.1±3.1
20:4(6)	3.3±1.8	0.8±0.5	0.3±0.3	0.7±0.5
20:5(3)	3.0±0.7	5.8±2.2	5.9±1.8	2.7±1.0
22:4(6)	5.9±1.7	10.8±8.2	9.3±3.4	8.2±5.0
22:5(6)	1.2±0.8	0.8±0.8	5.5±3.3	0.6±0.6
22:5(3)	3.3±2.1	3.7±1.7	4.5±2.9	1.6±0.6
22:6(3)	4.0±2.4	3.9±1.8	5.0±3.2	1.6±0.7
Σ saturates[a]	37.7	41.6	36.1	42.2
Σ PUFA	45.5	49.8	54.1	42.0
Σ MUFA	12.6	8.1	6.1	15.1
UI[b]	164.2	191.5	212.1	141.6
Σ n-9[c]	12.8	6.0	9.9	9.0
Σ n-6	31.0	32.5	33.4	33.0
Σ n-3	11.2	16.0	15.3	5.6
Σ(n6/n3)	2.8	2.0	2.2	5.9

[a]Includes fatty acids from C14:0 to C22:6(3).

[b]UI = Σ([a][b]), where 'a' is the percent of each unsaturated fatty acid and 'b' is the number of double bonds for that particular fatty acid

[c]Includes C18:1(7).

Table 3.12: Fatty acid distribution of diacyl phosphatidylethanolamine from the proximal small intestine. Data is given as mean (±SEM) on purified alkenyl fractions. Data is given as area% rather than mol%. T & SFO = tallow and safflower oil diet; SFO = safflower oil diet; T & SFO & DHA = tallow and safflower oil and docosahexaenoic acid diet; T & SFO & EPA = tallow and safflower oil and eicosapentaenoic acid diet. Numbers within a row with different superscripts are significantly different ($p<0.05$). Numbers in brackets at the top of each column indicate the number of rats in each group.

Fatty Acid	*Diet Fat Fed*			
Fatty Acid	T & SFO (6)	SFO (6)	T & SFO & DHA (7)	T & SFO & EPA (6)
16:0	5.4±1.8[a]	5.0±1.6[a]	3.0±1.4[a]	16.1±3.8[b]
16:1(5)	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.1
18:0	31.9±3.8	30.2±4.2	42.3±4.7	33.3±3.7
18:1(9&7)	9.4±2.0[a]	4.5±0.5[b]	5.8±1.8[ab]	7.9±0.8[ab]
18:2(6)	3.2±3.8	4.1±1.8	3.2±1.2	4.2±0.6
18:3(6)	5.3±2.3	5.4±1.3	3.9±2.1	3.6±1.1
18:3(3)	0.1±0.1	0.0±0.0	0.0±0.0	0.3±0.2
18:4(3)	0.6±0.4	0.0±0.0	0.0±0.0	0.6±0.4
20:3(9)	0.7±0.4	2.8±1.6	2.3±0.7	3.7±1.3
20:3(6)	2.8±1.1	2.8±1.4	2.8±2.4	1.6±1.5
20:4(6)	9.2±3.4[a]	3.8±1.6[ab]	5.0±2.3[ab]	1.1±0.6[b]
20:5(3)	1.6±0.8	3.2±1.3	6.1±2.4	3.7±1.5
22:4(6)	4.2±1.9	9.0±5.3	4.7±2.9	3.2±2.5
22:5(6)	6.6±2.6	10.1±4.9	2.2±0.8	0.7±0.4
22:5(3)	0.9±0.4	3.0±0.9	2.8±1.6	3.3±0.8
22:6(3)	3.1±1.9	1.8±0.6	6.0±2.5	3.0±1.6
Σ saturates[a]	45.0	45.3	52.2	57.3
Σ PUFA	44.3	46.0	39.0	29.0
Σ MUFA	10.6	6.4	8.9	13.0
UI[b]	175.8	191.1	172.6	125.1
Σ n-9[c]	11.2	8.8	11.2	15.8
Σ n-6	37.3	35.2	21.8	14.4
Σ n-3	6.3	8.0	14.9	10.9
Σ(n6/n3)	5.9	4.4	1.5	1.3

[a]Includes fatty acids from C14:0 to C22:6(3).

[b]UI = Σ([a][b]), where 'a' is the percent of each unsaturated fatty acid and 'b' is the number of double bonds for that particular fatty acid

[c]Includes C18:1(7).

3.2 Discussion

Dietary fat is a determinant in the phospholipid fatty acid composition and thus the biochemical function of various membranes [16, 89, 47]. Phospholipids are the major structural lipid of all biological membranes. Polyunsaturated fats are the structural components of membrane phospholipids and they determine the physicochemical properties of enterocyte membranes [29]. They also serve as substrates for eicosanoid synthesis [29]. Ether phospholipids constitute a major part of the total phospholipid in mammalian tissue [15, 13]. It is known that a high proportion of ether phospholipids contain arachidonic acid at the *sn–2* position of the glycerol backbone and that diet can alter arachidonic acid levels in the intestinal mucosa [3]. The experiment presented in this thesis was designed to examine if arachidonic acid availability influenced ether phospholipid biosynthesis.

3.2.1 Effect of Diet on Mucosal Tissue Weights

The EPA dietary group produced lower wet weight of mucosa from the proximal small intestine compared to the other three dietary groups (see Table 3.4). It is not known why this is the case since, by week four, the the body weights and the amount of food consumed by each group was similar. Actually the EPA diet produced the heaviest rats and so the discrepancy may be due to errors in scraping. No significant differences between diets were found in mucosal weights of the distal small intestine or the colon.

Overall the average weights of the mucosa were expected if compared to an earlier study by Garg et.al. [30] but they were much lower than those found by Yamada [98].

Yamada et.al. considered the proximal and distal small intestine together whereas in the study by Garg et.al. and in the present study each segment was considered individually. It should be noted that the mucosal weights in the present study were similar to those found in rats that had been fasted for twenty–four hours [30]. The rats in this experiment were fasted overnight before being sacrificed, (16 hours), and so it makes sense that the mucosal weights are similar to those found by Garg et.al.

3.2.2 Effect of Diet Treatment on the Phospholipid Composition of the Mucosa

Dietary fat had no significant effect on total phospholipid produced. Several studies have examined the total phospholipid content and composition of intestinal mucosa [19, 8, 87, 20, 27, 31, 30, 98, 50]. The present study is in agreement with others which found that the proximal small intestine contains more total phospholipid than the distal small intestine [30, 31, 87, 8]. However the present study obtained the most phospholipid from the colon which is in contrast to a study done by Brasitus et.al. [9]. This can be explained by the fact that the present study considered the entire colon intact while Brasitus et.al. considered only the basolateral membranes (BLM) of the colon.

Some studies on the phospholipid composition of the small intestine considered the mucosa as a whole [98] while others divided it into brush border membranes (BBM) and microvillus membranes [19, 8, 20, 50, 87, 27, 31, 30]. With the exception of three [31, 30, 27] all the above studies found PC to be the major phospholipid in the small intestine which is in agreement with the present study.

Phosphatidylcholine can be synthesised in various tissues by the following three

pathways:

1. acylation of lysoPC to PC [53];

2. a 'de novo' pathway which involves a reaction of a diacylglycerol with CDP–choline. This pathway is catalysed by cholinephosphotransferase [97, 51];

3. the enzymatic conversion of PE to PC by methylation [10, 21].

It is well established that the first two pathways exist in the rat small intestinal epithelial cell [19]. The 'de novo' pathway is thought to be the dominant one [39]. The third pathway could not be found in intestinal cells by Bremer et.al. [10], however, it has recently been found to exist in small intestine plasma membranes of the rat by Dudeja et.al. [19]. It is still not known whether one or two methyltransferase enzymes are involved.

Methyltransferase activity can be altered by diet [39]. Diets containing high levels of n–6 fatty acids produce a membrane lipid with a high n–6/n–3 ratio which in turn increases phosphatidylethanolaminemethyl transferase activity [39]. This may account for the fact that more PC than PE was recovered in this study. The T&SFO dietary group produced the greatest amount of PC in the proximal small intestine. This group also had the largest n–6/n–3 ratio in the diacyl subclass which would support the theory that the n–6/n–3 ratio is an important regulator of the transmethylase activity [39].

Diet altered the amount of PC but not PE produced in the proximal small intestine and in the colon. It is known that the more unsaturated the diet the less PC found in the enterocyte microvillus membrane preparation of the small intestine

[8]. In the present study the T&SFO diet was the most saturated and produced the most PC in the proximal small intestine and in the colon. While this agrees with the study by Brasitus et.al. it is contrary to the results found in other studies [50, 20]. This discrepancy may be explained by the fact that both Keelan et. al. and Dudeja et. al. looked only at the BBM while the present study considered the mucosa as a whole.

3.2.3 Effect of Diet on Alkenyl and Diacyl Subclasses of the PE and PC Fraction of the Mucosal Cells

In most mammalian tissue the predominant plasmalogen is the PE plasmalogen [18, 97, 68, 81, 83, 44, 6, 77]. There are some exceptions to this but they are mainly in the myocardium of some birds and mammals [38]. According to Strum the PC plasmalogen is also found in significant quantities in striated and smooth muscle of mammals [77]. The few studies that have examined rat intestine (mucosa, smooth muscle, or entire small intestine) all found little if any plasmalogen content of the PC fraction [98, 66, 27]. In the present study the amounts PE and PC plasmalogens are approximately equal. Some of these discrepancies could be due to differences in the sex of animals, the preparations examined (ie microvillus membranes and BBM), or the separation method used to distinguish between plasmenylcholine and plasmenylethanolamine [65, 66, 64, 27].

It is known that the ethanolamine plasmalogens are biosynthesised by the desaturation of 1–O–alkyl–2–acyl–*sn*–glycero–3–phosphoethanolamine catalysed by the 1–alkyl–2–acyl–*sn*–glycero–3–phosphoethanolamine Δ–1–desaturase enzyme [76]. However this alkyl ether desaturase enzyme is specific for plasmanyl ethanolamine and

so far an analogous pathway for the formation of plasmenylcholine has not been observed [77, 97, 6]. To date the mechanism by which the choline plasmalogen is synthesised in biological systems is not well understood.

Recently Blank et.al. demonstrated that in HL–60 cells a significant amount of plasmenylcholine is synthesised by three pathways [6]:

1. from plasmenylethanolamine via a series of sequential steps involving phospholipase A2, lysophopholipase D, acyltransferase and cholinephosphotransferase;

2. by direct base exchange of choline for ethanolamine in the PE plasmalogen;

3. by exchange of choline for the ethanolamine of plasmenylethanolamine by steps involving phospholipase C hydrolysis of plasmenylethanolamine and cholinephosphotransferase.

Strum et.al. demonstrated that, at least in Madin–Darby Canine kidney cells, phospholipase D is not involved in the synthesis of plasmenylPC. They further suggest that lysophospholipase C mediates the conversion by producing alkenylglycerol from alkenyl–lyso–*sn*–glycero–3–phosphoethanolamine. Plasmenyl PC is formed subsequently by the condensation of this alkenylglycerol with CDP–choline in a 'de novo' type pathway which involves cholinephosphotransferase [77]. Most recently it has been demonstrated that it is the same cholinephosphotransferase enzyme that catalyses the production of phosphatidylcholine that is also responsible for the synthesis of plasmenylcholine [97]. It has been shown that the turnover rate of PC increases significantly when diet fat is absorbed suggesting that diet has some regulatory effect on cholinephosphotransferase [37].

In the present study it was shown that the T&SFO diet but not the SFO diet produced significantly more of the alkenyl subclass of PC in the distal small intestine than the other three diets. The T&SFO diet is unique in that it has the lowest content of polyunsaturated fatty acids and the highest amount of monounsaturated fatty acids (see Table 3.1). It is possible that this combination has a stimulatory effect on either lysophospholipase C or on cholinephosphotransferase.

It is also known that alkylacylglycerophosphoryl ethanolamine desaturase, the enzyme that is responsible for the production of plasmenylethanolamine from alkylacyl–GPE , in tumor cells is not effected by diet [12]. As far as I know this has never been demonstrated in healthy cells and it may be that the T&SFO combination, or the PUFA/MUFA ratio, has a slight inhibitory effect on this enzyme thereby lowering the ethanolamine plasmalogen content of healthy enterocytes. This, along with an upregulation of lysophospholipase C or cholinephosphotransferase, may explain the increase in the alkenyl PC seen in this study.

Finally, this increase in plasmenylcholine would be responsible for less fluidity in the cellular membranes in the mucosa. Membrane preparations isolated from rats fed unsaturated fatty acid diets had enhanced fluidity when compared to preparations obtained from rats fed saturated fatty acid diets [8]. The greatest enhancement of membrane fluidity occurred in the distal enterocyte microvillus membranes. The T&SFO diet had the greatest quantity of saturated fat and the lowest quantity of PUFA. This combination may have had an effect on the enterocyte membrane lipid composition thus altering its fluidity. This altered fluidity, thought to effect various membrane functions [16], may have effected the enzymes responsible for the synthesis of the PC plasmalogen. Radioactive studies following the pathway of synthesis for

the alkenyl subclass of PC, would need to be done to determine if dietary fat did indeed effect these enzymes.

3.2.4 Fatty Acid Composition of the Alkenyl and Diacyl Subclass of PE and PC

Diet significantly effected the fatty acid composition of both the alkenyl and diacyl subclasses of PC in the distal small intestine (see Tables 3.9 and 3.10) and of PE in the proximal small intestine (see Tables 3.11 and 3.12). Generally, as the saturation of the dietary fat decreased so did the saturation of the fatty acids found in the diacyl subclasses of PC and PE and in the alkenyl subclass of PC. No correlation between degree of unsaturation in the diet and the UI of the tissue was observed in the alkenyl subclass of PE. The low level of saturated fatty acid in the mucosa of rats fed low levels of saturated fatty acids was mostly accounted for by the reduction of C16:0. The greatest reduction of C16:0 was found in the diacyl subclass of PC.

The major fatty acyl constituents were the same in the alkenyl and diacyl subclasses of PC and PE except that the diacyl contained more C18:2(6). With the exception of the C18:2(6) content in the alkenyl subclass of PC these results are in agreement with other studies examining various other tissues [71, 70, 94, 95, 30, 31, 29, 33].

The most surprising result was the low levels of C18:2(6) produced in the alkenyl subclass of PC by the safflower oil diet (see Table 3.10). All the diets resulted in low levels of alkenyl C18:2(6) but the SFO diet caused much lower values than the other three diets. The low level of C18:2(6) did not result in low levels of C18:3(6). Although the C18:2(6) levels are also low in the diacyl class they are not as low

as in the alkenyl class. The low level of linoleic acid without a similar reduction in C18:3(6) is not so surprising once one takes into account that the rats had been fasted for at least 16 hours before sacrifice. The low level of C18:2(6) without a concomitant reduction in C18:3(6) or C20:4(6) suggests an increase in the activity of the Δ^6 or the Δ^5 desaturase enzyme [31]. It has been shown that although the Δ^6 desaturase activity is decreased in the liver by fasting [17, 11] it is stimulated under the same conditions in the intestine [29, 33]. The fact that no decrease in C18:3(6) or C20:4(6) was caused by the SFO or the T&SFO diets supports the idea that fasting stimulates Δ^6. If Δ^6 was not stimulated then low C18:2(6) levels would be expected to occur with low C18:3(6) levels but this was not the case.

It is interesting that the DHA diet produced low levels of C18:3(6) and C20:4(6) in both subclasses of PC (see Figure 3.1). This may suggest that DHA has an inhibitory effect on the Δ^5 desaturase enzyme. The EPA, on the other hand, while also causing a decrease in C20:4(6), caused a build up of C18:3(6). This suggests that while DHA may inhibit the Δ^5 desaturase enzyme, EPA seems to inhibit Δ^5 but stimulate the Δ^6 enzyme. This is in contrast to a study done which claims that $\omega 3$ fatty acids have no effect on the intestinal desaturase enzymes [29] but rather have their influence at the acyltransferase enzyme which is known to have a greater affinity of EPA and DHA than for arachidonic acid [30]. This theory is also plausible and it also helps to explain why in the present study the DHA diet caused a build up of C22:6(3) in both the alkenyl or diacyl subclass of PC. The important thing to note is that both the EPA and DHA diets cause a decrease in C20:4(6) in both subclasses (see Figure 3.2). In the alkenyl subclass the decrease is only significant in the the animals fed DHA. The DHA diet caused a significant reduction in levels of

the alkenyl subclass of PC compared to the EPA diet but not compared to the SFO diet. This may suggest that DHA is more efficient than EPA at limiting arachidonic acid availability and therefore at affecting ether phospholipid synthesis. It is possible that DHA has a stronger affinity for the Δ^5 desaturase enzyme than does EPA and thus is more efficient at blocking C20:4(6) synthesis. However, if this were true one would expect to find lower levels of C20:4(6) in the tissue of the animals fed DHA when compared to those fed EPA. This was not the case which might suggest that other regulatory factors are involved in ether phospholipid biosynthesis than diet.

When one designs experimental diets it is important to consider the n6/n3 ratio of each diet because of the competition that exists between these two families of fatty acids for enzymes involved in their metabolism and incorporation into phospholipids [33, 40]. The fish oil diets in this study, (both the DHA and EPA), had significantly lower n6/3 ratio, (1.4 and 1 respectively), than the other two diets (32.3 and 249). A slight build up of C20:5(3) was only found in the EPA diet group in both PC subclasses. In the alkenyl subclass there was a significant increase in C22:5(3) compared to the T&SFO and SFO diets and in the diacyl subclass C22:5(3) was significantly increased by the DHA and EPA diets compared to the T&SFO diet. The lower n6/n3 ratio may allow for better conversion of C18:3(3) to C20:5(3) [95] and of C20:5(3) to C22:5(3). This ratio may also be responsible for the regulation of the retroconversion from C22:6(3) to C22:5(3) which seems to have occurred in both PC subclasses of the animals fed fish oil.

Unexpectedly, in the diacyl and alkenyl subclasses of PC the fish oil diets caused a decrease in C22:6(3) fatty acids. It is possible that the conversion of C22:5(3) to C22:6(3) is depressed or retroconversion is activated under conditions where dietary

n6/n3 ratios are low. It may be that this low ratio has an inhibitory effect on Δ^4 desaturase. This result was also seen in human platelets under the same dietary conditions [95].

It has recently been suggested that a Δ^4 desaturase enzyme is not involved in the desaturation of C22:5(3) to C22:6(3) [94]. There is evidence to suggest that C22:5(3) is metabolised first to C24:5(3) and then to C24:6(3) and finally, by a retroconversion reaction, to C22:6(3) [94, 93]. An analogous pathway has also been suggested for the C22:4(6) conversion to C22:5(6). The same study indicated that C22:4(6), C22:5(6), C22:5(3) and C22:6(3) are all good substrates for retroconversion yielding either arachidonic acid or eicosapentaenoic acid. In the present study it would appear that retroconversion is effected by diet since, in both the alkenyl and diacyl subclasses of PC, the EPA diet seemed to cause the greatest amount of retroconversion from C22:5(3) to C20:5(3) and the DHA diet seemed to cause the retroconversion of C22:6(3) to C22:5(3). Much research needs to be done to determine the precise mechanisms and regulatory controls for the interconversion of the n3 and n6 fatty acids.

Finally, in the animals fed EPA there seemed to be a significant build up of C20:3(9) in the diacyl PC and a slight increase of C20:3(9) in alkenyl PC. This fatty acid is usually an indicator of essential fatty acid deficiency [54, 23]. It was believed in the present study that enough linoleic acid was added to each diet to prevent essential fatty acid deficiency. Recently a study done by Lands has suggested that 3en% of C18:2(6) is needed to eliminate the build up of Mead acid [54]. This same study showed that by using a n6/n3 ratio of one the C20:4(6) content in rat liver could be reduced by half. In the present study we saw a three to five fold reduction of

C20:4(6) in both subclasses caused by the fish oil diets. Even if the energy percentage of C18:2(6) added to each diet was enough to prevent essential fatty acid deficiency the fact that the EPA diet caused a build up of Mead acid suggests that the high level of n3 fatty acids, especially C20:5(3), in the EPA diet might out compete n6 fatty acids for the elongation and desaturation enzymes. This would effectively mimic essential fatty acid deficiency. Why this occurs in the EPA diet but not the DHA diet is unknown. Possibly these enzymes have a preference for C20:5(3).

It is known that C20:4(6), dihomo-γ-linolenate, EPA, DHA and Mead acid are good substrates for arachidonyl-CoA synthase, the enzyme thought to be required for eicosanoid precursor homeostasis [63]. It is possible that in the presence of high levels of EPA and arachidonic acid the enzyme will not recognize either and will selectively use C20:3(9). This would essentially mimic the condition where cells are starved for arachidonic acid. Certainly Mead acid build up was found in arachidonate-depleted rat polymorphonuclear leukocytes [70].

Fewer changes were observed in fatty acid composition of the alkenyl and diacyl subclasses of PE when compared to the PC fraction. The major fatty acyl constituents in both subclasses of PE were the same. The EPA diet caused a significant increase in C16:0 in diacyl PE which accounted for the fairly high saturated fat level observed.

The animals fed EPA produced significantly more alkenyl C18:2(6) in the PE fraction compared to those fed DHA but not compared to those fed T&SFO or SFO. This does not agree with an earlier study by Garg et. al., [33], however Garg examined the liver, not the intestine. It is known that fatty acid metabolism is regulated differently in the liver than in the intestine. The EPA fed animals also

produced less C18:3(6) compared to the DHA fed animals. This is the opposite result found in the PC fraction from the distal small intestine which suggests that fatty acid metabolism and esterification into phospholipids is regulated differently in the two segments of the small intestine [8].

The reduction of alkenyl and diacyl C18:2(6) in the PE fraction caused by the DHA diet occurs with a concomitant increase in C20:5(3) and C22:6(3). It is known that acyltransferase has a greater affinity for DHA and EPA than for arachidonic acid, [29, 30, 14], and this data suggests that it also has a greater affinity for DHA than for EPA.

C20:4(6) was reduced in alkenyl PE by both fish oil diets but the reduction was not significant when compared to the other two diets. This may simply be due to the large errors obtained in the values. The DHA diet caused the greatest reduction in arachidonic acid and also caused the greatest reduction in the alkenyl PE levels. This again suggests that DHA is more efficient at reducing C20:4(6) synthesis than is EPA and it has a greater regulatory control on ether biosynthesis.

In the PE diacyl subclass the major changes were observed in C16:0, C18:1(9&7) and C20:4(6). C20:4(6) was significantly reduced in the animals fed EPA compared to those fed T&SFO. With the exception of the change in C20:4(6) all of these changes are in contrast to those observed by Garg et. al. [33] but one must keep in mind that different tissues were examined.

Dietary C20:5(3) but not C22:6(3) caused a significant decrease in C20:4(6) in the diacyl subclass of PE. It has been suggested that C20:5(3) but not C22:6(3) has an inhibitory effect on the conversion of C20:3(6) to C20:4(6) [62]. This is consistent with the present experiment for the diacy subclass of PE but not PC. This difference

may be due to location (proximal vs distal). The reduction of C20:4(6) by EPA suggests that EPA exerts an inhibitory effect on the Δ^5 desaturase enzyme.

The PE data was difficult to assess. Differences expected to be significant were not but this may be due to the large errors in the data. The errors may be due to the age of the sample. Some of the sample may have oxidized due to the length of time it spent untouched. It would have been interesting to determine the extent of oxidation that occurred.

In conclusion, the present study, in agreement with other studies, [71, 24, 29, 50, 16, 87, 31, 64, 101, 30, 8], has shown that dietary fat can significantly effect the fatty acid composition of mucosal cells in the small intestine of rats. Research is needed to further delineate the exact mechanisms by which the effect occurs.

3.2.5 General Discussion and Further Research

Arachidonic acid and its metabolites are important precursors for eicosanoid production [15]. It is not yet known which phospholipids are sources of this potent inflammatory effector molecule [15]. The enterocyte lining of the small intestine is continually adapting to changes in nutrients due to diet [89]. These changes can effect the class and fatty acid composition of the phospholipid produced [88]. It has been shown that fish oils (EPA and DHA) can reduce the content of arachidonic acid in the tissue.

Ether phospholipids often contain a significant amount of arachidonic acid at their *sn*-2 position [34]. For this reason the present study was designed to test the hypothesis that arachidonic acid availability is the limiting factor in the production of ether phospholipids. This was accomplished by feeding rats diets rich in the fish oils eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are known to alter arachidonic acid metabolism. The colon and the mucosa of the proximal and distal small intestine were examined to determine total phospholipid content, phospholipid class (PE or PC), phospholipid subclass (alkenyl or diacyl) and the fatty acid composition of each subclass.

It was found that by altering arachidonic acid availability, the amount of total phospholipid was unchanged in each tissue. The amount of phosphatidylethanolamine was also unchanged however, in the proximal small intestine the proportion of the ether phospholipids was significantly altered by the two fish oil diets. The amount of alkenyl PE was significantly reduced in animals fed DHA and significantly increased in animals fed EPA. The phosphatidylcholine quantity was also increased in the

proximal small intestine but the proportion of ether phospholipids was altered only in the distal small intestine. The safflower oil (SFO) and the two fish oil diets caused significantly lower alkenyl phospholipids and higher diacyl phospholipids compared to the tallow and safflower oil diet (T & SFO). It was hypothesised that by limiting arachidonic acid availability through the altering of diet fat composition one would reduce the amount of ether phospholipid produced. This was the case for DHA in the proximal small intestine but not for EPA. In the distal small intestine DHA also caused a reduction in alkenyl C20:4(6) and the alkenyl subclass of PC but so did the EPA diet. The T&SFO diet also reduced alkenyl levels in PC but it did not reduce C20:4(6) levels. It was therefore concluded that while diet does have an effect it is not the only regulator of ether phospholipid biosynthesis and that DHA is a more potent regulator of C20:4(6) metabolism and ether phospholipid metabolism than is EPA. It should also be noted that, as expected, the distal and proximal small intestine seem to be subject to different regulatory controls.

One would need to do some radioactive studies to follow the pathway of ether phospholipid biosynthesis to see where the regulation occurs and exactly how diet plays a role. To obtain a better understanding one should also consider the alkylacyl ether phospholipid, and unfortunately it was not examined in the present study.

There is still much work that needs to be done in the area of ether phospholipid and plasmalogen biosynthesis. In the present study diet altered PC but not PE in the proximal small intestine and in the colon. It needs to be determined exactly which enzymes in the biosynthetic pathway of PC are altered by diet. One mechanism by which PC is produced is through PE via a PE methyltransferase enzyme [39, 10]. It needs to be determined how many methyltransferase enzymes are

involved in the conversion of PE to PC [39]. The regulation of this (these) phosphatidylethanolaminemethyltransferase enzyme(s) needs further investigation.

An enzyme that is more effective in producing PC and plasmenylPC is cholinephosphotransferase [6, 77, 97]. Whether or not this enzyme is regulated by diet also needs to be determined.

Some research has been done to delineate the regulatory control diet has on enzymes involved in fatty acid elongation and desaturation [31, 29, 30] but it seems very little research has been done on other enzymes involved in fatty acid metabolism and esterification into phospholipids. These enzymes also need to be studied if we are to have a full understanding of the effects of diet on membrane phospholipid composition. The n3/n6 ratio in the diet also needs to be examined to determine which ratio has the optimal effect on the enzymes involved in ether phospholipid biosynthesis [33].

Finally, the role that ether phospholipids play in disease and aging would be interesting to assess. It is important to examine how enzymes involved in fatty acid metabolism and phospholipid and plasmalogen biosynthesis are affected by diabetes mellitus, intestinal resection, cystic fibrosis, aging and inflammatory bowel disease [29]. Once this is determined we can begin to evaluate nutritional means to alleviate some of the symptoms of these altered states.

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